Role of DNA methylation in common disease: analysis of two asthma-associated regions

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August, 2017

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

DEDICATION

To my lovely husband AbdulAziz Attuwaijri who always stood by me

ABSTRACT

Two chromosomal regions, 5q31 and 17q21, are both among the best replicated asthma-associated regions from genome-wide association studies (GWAS) and share certain characteristics.They are among the highest genome-wide significance allele-specific expression differences: in the 17q region, the childhood asthma-associated allele shows increased expression of gasdermin B (*GSDMB*) and *ORMDL* sphingolipid biosynthesis regulator 3 (*ORMDL3*) genes and in the 5q31 region the asthma-associated allele shows increased expression of the solute carrier family 22 (organic 3 cation/carnitine transporter) member 5 (*SLC22A5*) genes. This suggests that cis-regulatory mechanisms may influence disease development in these regions. Among children, the prevalence of asthma is higher in boys than girls, 5q31 and 17q21 regions show sex specificity of genetic association: the association with asthma is stronger in males than females, and both are associated with several autoimmune disorders such as asthma and Crohn's disease, among different populations. We hypothesize that variation in DNA methylation at regulatory elements within asthma-associated genomic regions 5q31 and 17q21 may acts as a modifier of the effect of genotype on phenotype. To better understand the relationship between genetic variation in these regions, DNA methylation, and predisposition to asthma, we established DNA methylation profiles of 13 genes. We used sodium bisulfite sequencing methylation assay to examine the effects that genotype has on DNA methylation in the peripheral blood cells from individuals in the Saguenay-Lac-Saint-Jean (SLSJ) asthma familial collection and lymphoblastoid cell lines (LCLs). We found a single regulatory region, the *zona pellucida* binding protein 2 (*ZPBP2*) promoter, which showed a statistically significantly higher methylation level in females compared to males. The local genotype influenced methylation levels of *SLC22A5*, *ZPBP2* and gasdermin A (*GSDMA*) promoter regions. The genotype had a dominant effect on *SLC22A5, ZPBP2* and *GSDMA* methylation, with lower methylation levels in individuals that carry the asthma-predisposing alleles. To understand whether the moderate change in DNA methylation leads to a change in gene expression levels in 5q31 and 17q21, the effect of treatment with DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-aza-dC) on expression and promoter methylation of genes in 5q31 and 17q21 in the human airway epithelium cell line, NuLi-1 and embryonic kidney epithelium cell line, HEK293T/17 was assessed. We found that *SLC22A5* and *GSDMA* were upregulated after 5-aza-dC treatment in both cell lines. *ZPBP2* expression was increased in NuLi-1 but remained silent in HEK293T/17, while *ORMDL3* showed upregulation in HEK293T/17 but not NuLi-1. Increased

expression of *ZPBP2* and *GSDMA* was accompanied by a reduction in promoter methylation, whereas no change in *SLC22A5* promoter methylation levels was observed. Furthermore, we noticed modification in allelic expression of *ZPBP2* and *ORMDL3*, suggesting that each region may react in uniquely to 5-aza-dC.

We hypothesized that sex-specific differences in DNA methylation levels at the *ZPBP2* promoter resulted from the dosage of the sex chromosomes. Thus, *ZPBP2* DNA methylation was analyzed using the sodium bisulfite sequencing assay in DNA samples from fibroblast cell lines derived from individuals with different sex phenotypes and sex chromosome dosages. No significant influence of the sex phenotype was detected. However, a significant positive correlation was found between *ZPBP2* methylation levels and the number of X chromosomes. Our results suggest that the presence of more than one X in the absence of the sex-determining region Y (*SRY*) gene is associated with higher methylation levels.

RÉSUMÉ

Deux régions chromosomiques, 5q31 et 17q21, font partie des régions associées à l'asthme les mieux répliquées des études d'association pangénomique (GWAS) et partagent certaines caractéristiques. Ces régions ont les plus grandes différences significatives d'expression génique associée aux allèles au niveau du génome : dans la région 17q, l'allèle associé à l'asthme chez l'enfant montre une augmentation de l'expression des gènes gasdermin B (*GSDMB*) et «Régulateur de biosynthèse des sphingolipides ORMDL 3» (*ORMDL3*); alors que c'est le gène «famille de support de soluté 22 (transporteur de 3 cation / carnitine organique) membre 5» (*SLC22A5*) qui est augmenté dans la région 5q31. Ceci suggère que les mécanismes régulés en *cis* peuvent influencer le développement des maladies dans ces régions. Chez les enfants, la prévalence de l'asthme est plus élevée chez les garçons que chez les filles. Les régions 5q31 et 17q21 montrent une association génétique spécifique liée au sexe: l'association avec l'asthme est plus forte chez les males que chez les femelles, et les deux sont associés à de nombreux troubles auto-immunes, tels que l'asthme et la maladie de Crohn, dans les différentes populations. Nous posons l'hypothèse que la variation de la méthylation de l'ADN des éléments régulateurs dans les régions génomiques associées à l'asthme, 5q31 et 17q21, peut agir comme modificateur des effets du génotype sur le phénotype. Afin de mieux comprendre la relation entre la variation génétique de ces régions, la méthylation de l'ADN et la prédisposition à l'asthme, nous avons établi les profils de méthylation d'ADN de 13 gènes. Nous avons utilisé des essais de méthylation au bisulfite de sodium et de séquençage pour examiner les effets que le génotype a sur la méthylation de l'ADN dans les cellules du sang périphérique provenant d'individus de la collection familiale asthmatique du Saguenay-Lac-Saint-Jean (SLSJ) et les lignées de cellules lymphoblastoïdes (LCLs). Nous avons identifié une seule région régulatrice, le promoteur de «*zona pellucida* binding protein 2» (*ZPBP2*), lequel a montré un plus haut niveau statistiquement significatif de la méthylation chez les femelles comparativement aux males. Le génotype local a influencé les niveaux de méthylation des promoteurs de *SLC22A5*, *ZPBP2* et gasdermin A (*GSDMA*). Le génotype avait un effet dominant sur la méthylation de *SLC22A5*, *ZPBP2* et *GSDMA*, avec des niveaux de méthylation plus bas chez les individus porteurs des allèles de prédisposition à l'asthme. Pour comprendre comment le changement modéré dans la méthylation d'ADN mène à un changement dans les niveaux de l'expression des gènes dans les régions 5q31 et 17q21, nous avons examiné les effets d'un traitement à l'inhibiteur de la méthyltransférase de l'ADN 5-aza-2'-

déoxycitidine (5-aza-dC), lequel induit la déméthylation de l'ADN, sur l'expression et la méthylation des promoteurs des gènes situés aux chromosomes 5q31 et 17q21 dans les lignées de cellules humaines épithéliales des voies aériennes (NuLi-1) et du rein embryonnaire (HEK293T/17). Nous avons trouvé que *SLC22A5* et *GSDMA* étaient régulés positivement suite à un traitement au 5-aza-dC dans les deux lignées cellulaires. L'expression de *ZPBP2* était augmentée dans les cellules NuLi-1 mais restait silencieuse dans les cellules HEK293T/17, tandis qu'*ORMDL3* montrait une régulation positive dans les HEK293T/17 mais pas dans les NuLi-1. Les expressions augmentées de *ZPBP2* et de *GSDMA* étaient accompagnées par une diminution de la méthylation du promoteur, alors qu'aucun changement des niveaux de méthylation du promoteur de *SLC22A5* n'a été observé. De plus, nous avons remarqué une modification dans l'expression allélique de *ZPBP2* et *ORMDL3*, suggérant que chaque région peut réagir de façon unique au 5-aza-dC.

Nous avons émis l'hypothèse que les différences liées au sexe dans les niveaux de méthylation de l'ADN du promoteur de *ZPBP2* étaient dues au dosage des chromosomes sexuels. Ainsi, la méthylation d'ADN de *ZPBP2* a été analysée par essais de bisulfite de sodium et de séquençage dans des échantillons d'ADN de lignées cellulaires de fibroblastes provenant d'individus avec des différences du phénotype sexuel et du dosage des chromosomes sexuels. Aucune influence significative du phénotype sexuel n'a été détectée. Toutefois, une corrélation significativement positive a été trouvée entre les niveaux de méthylation de *ZPBP2* et le nombre de chromosomes X. Nos résultats suggèrent que la présence de plus d'un chromosome X en absence du gène *SRY* est associée à des niveaux plus élevés de méthylation.

CONTENTS

LIST OF ABBREVIATIONS

5-aza-dC: 5-aza-2'-deoxycytidine

5mC: 5-methylcytosine

AAA: abdominal aortic aneurysm

ACSL3: acetyl-CoA synthetase long-chain family member 3

ADHD: attention-deficit hyperactivity disorder

AHR: airway hyperresponsiveness

Alu: arthrobacter luteus

AML: acute myeloid leukemia

ASD: autism spectrum disorders

ASM: of allele-specific DNA methylation

ATRX: alpha thalassemia/mental retardation syndrome, X-linked

BM: bone marrow

BPA: bisphenol A

CACYBP: calcyclin-binding protein

CAV1: Caveolin-1

CD4: cluster of differentiation 4

CD8: cluster of differentiation 8

CD45: cluster of differentiation 45

CD48: cluster of differentiation 48

cDNA: complementary DNA

CEPH: centre d'etude du polymorphisme humain

CG (or CpG): 5' – cytosine – phosphate – guanine – 3'

CGI: CpG island

CLDN11: Claudin 11

CLLORF52: chromosome 11 open reading frame 52

c-MYC: cellular myelocytomatosis

CSF2: colony-stimulating factor 2

CSF3: colony-stimulating factor 3

CTCF: CCCTC-binding factor

DNA: deoxyribonucleic acid

DNMT1/ Dnmt1: DNA methyltransferase 1

DNMT3A/Dnmt3a: DNA methyltransferase 3A

DNMT3B/Dnmt3b: DNA methyltransferase 3B

DNMT3L/Dnmt3l: DNA methyltransferase 3 like

DNMTs: DNA methyltransferase enzymes

EAE: experimental autoimmune encephalomyelitis

EBV: Epstein–Barr virus

eDMR: enhancer differentially methylated region

EDTA: ethylenediaminetetraacetic acid

ESCs: embryonic stem cells

Esr1: estrogen receptor 1

FBXL5: f-box and leucine-rich repeat protein 5

FCG: four core genotypes

FDR: false-discovery rate

FRMD4A: FERM Domain Containing 4A

FEV_1: forced expiratory volume 1

FOXP3: forkhead box P3

ELF5: factor E74-like factor 5

eQTL: expression Quantitative trait loci

GLT-1: glutamate transporter subtype 1

GSDMA: gasdermin A

GSDMB: gasdermin B

GWAS: genome-wide association study

H3K4: histone H3 lysine 4

H3K4me3: histone H3 lysine 4 trimethylated

H3K9ac: histone H3 lysine 9 acetylated

HAP: haplotype

HATs: histone acetyltransferases

hCD2: human cluster of differentiation 2

HDAC: histone deacetylase

HGSNAT: heparan-α-glucosaminide N-acetyltransferase

HRV: human rhinovirus

IAP: intracisternal A particle

IBD: inflammatory bowel disease

ICM: inner cell mass

IFN: interferon

IFNα: interferon alpha

IFNβ: interferon beta

IFNγ: interferon gamma

IgE: Immunoglobulin E

Igf2: insulin-like growth factor 2

IKZF3: IKAROS family zinc finger 3

IL3: Interleukin 3

IL5: Interleukin 5

IL9: Interleukin 9

IL13: Interleukin 13

IL17: Interleukin 17

IL33: Interleukin 33

iNKT: invariant natural killer T

IRF1: regulatory factor 1

Kb: kilobase

KDM6A: Lysine-specific demethylase 6 A

KDM5C: Lysine-specific demethylase 5 C

LCLs: lymphoblastoid cell lines

LINE-1: long interspersed nuclear element

LPS: lipopolysaccharides

MAF: minor allele frequency

MAO A: monoamine oxidase A

MBD2: methyl-binding protein MBD2

MDS: myelodysplastic syndrome

MECP2: methyl-CpG-binding protein 2

MS: multiple sclerosis

MZ: monozygotic twins

NANOG: nanog homeobox

NO: nitric oxide

OCTN: organic cation transporter

ORMDL3/Ormdl3: orsomucoid like 3

P4HA2: prolyl 4-hydroxylase, alpha polypeptide II

PBCs: peripheral blood cells

PBMCs: peripheral blood mononuclear cells

PCDH20: protocadherin-20

PCR: polymerase chain reaction

PD: Parkinson's disease

PDLIM4: PDZ and LIM domain 4

PDYN*:* prodynorphin

PIBD: pediatric inflammatory bowel disease

POA: preoptic area

POMC: pro-opiomelanocortin

PSCD: primary systemic carnitine deficiency

qPCR: quantitative polymerase chain reaction

RNA: ribonucleic acid

RSV: respiratory syncytial viral

RT-qPCR: quantitative reverse transcriptase polymerase chain reaction

SAM: S-adenosylmethionine

SCMH1: sex comb on midleg, drosophila, homolog of 1

SCS: sex-chromosome-complement-sensitive

shRNA: small hairpin RNA

siRNA: short-interfering ribonucleic acids

SLC22A4: (organic cation/zwitterion transporter), member 4

SLC22A5: Solute Carrier Family 22 Member 5

SLE: systemic lupus erythematosus

SLSJ: Saguenay-Lac-Saint-Jean

SNP: single nucleotide polymorphism

Sp1: specificity protein 1

Sp3: specificity protein 3

SRY/Sry: sex-determining region Y

SS: Sjogren's syndrome

SSRI: selective serotonin reuptake inhibitor

T-DMR: tissue-specific differentially methylated region

TDT: transmission disequilibrium test

Teff: effector T cell

TH1: T helper 1

TH2: T helper 2

TNFα: necrosis factor-alpha

Treg: regulatory T cell

TSC: trophoblast stem cells

TSLP: thymic stromal lymphopoietin

TSS: Transcription start site

UCSC: University of California at Santa Cruz

WDR36: WD Repeat Domain 36

XCI: X chromosome inactivation

XLID: X-linked intellectual disability

Xp: paternal X

ZPBP2: zona pellucida binding protein 2

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ACKNOWLEDGMENTS

I would like to humbly acknowledge all those who helped me to successfully complete this research.

First and foremost, I would like to thank the Almighty God who was always with me and provided me with all the strength I needed to move forward in life. I would like to express my profound sense of reverence and gratitude to my supervisor Dr. Anna Naumova, for her day to day supervision, advice and guidance on this research project. She provided me with unflinching encouragement and an ideal environment to perform scientific research. I would like to thank my supervisory committee members Dr. Jacquetta Trasler and Dr. Danuta Radziochfor for their helpful comments and insights on my project. In addition, I would also like to thank Dr. Simon Rousseau (McGill University) for providing us with the human airway epithelium cell line, NuLi-1.

I am deeply thankful for the daily technical and social support from Dr. Naumova lab members: Sanny Moussette, Pamela Stroud, Dr. Hamid-Reza Kohan-Ghadr, Dr. Nasser Fotouhi-Ardakani, Jacky Leung, Victoria Chiwara, and Bianca Ho. I would also like to thank the Department of Human Genetics at McGill University.

Words fail me when trying to show appreciation for my husband Abdulaziz Attuwaijri, the love of my life. He deserves special mention for his unconditional support, prayers, love, persistence and encouragement to achieve my best. “Thank you for always believing in me “.

I would also like to express my gratitude to my Scholarship Sponsor King Abdulaziz Medical City in Riyadh for funding my study.

Last but not least, I would like to take this opportunity to express my love and profound gratitude to my beloved mother for giving me life, for educating and providing me with unconditional support to follow my dreams, and to my daughters, Jodie and Laura Attuwaijri for their ever-lasting smiles, which relieved me whenever I was frustrated. I also owe special thanks to my dearest friend Vafa Keser for being there whenever I needed her.

THESIS FORMAT

This is a traditional monograph style thesis, which conforms with the "Guidelines for Thesis Preparation" of the Faculty of Graduate Studies and Research at McGill University. This thesis is comprised of four chapters. Chapter one is a literature review and introduction that recapitulate the background material for this thesis and state the scientific questions and objectives of my research. Chapter two describes the materials and methods that were used in my project. Chapter three contains results that were published in *Human Genetics*, 2012 (PMID: 22271045), 2013 (PMID: 23546690), the *Journal of Medical Genetics*, 2016 (PMID: 26671913) and *PloS One*, 2017 (PMID: 28241063). Chapter four contains detailed discussion of my findings. Chapter five provides conclusions and possible future directions for the project. References for all chapters are provided at the end of the thesis using EndNote X7 software.

CONTRIBUTIONS OF AUTHORS

I designed, validated and analyzed DNA methylation assays for the, *GSDMB* promoter and exon 1 of isoform 2, *GSDMA* and *CSF3* promoter regions and analyzed DNA methylation patterns in the rs4795397 region, and promoters of *IKZF3, ZPBP2* and *ORMDL3* in DNA samples from CEPH HapMap panel I LCLs and whole blood from SLSJ subjects using the sodium bisulfite sequencing methylation assay

I designed, validated and analyzed DNA methylation assays for 8 regulatory regions *SLC22A5, SLC22A4, P4HA2, IRF1, IL3, CSF2, PDLIM4* and *IL5* in 5q31 in DNA samples from CEPH HapMap panel LCLs using the sodium bisulfite sequencing methylation assay. I analyzed pyrosequencing results for *SLC22A5, GSDMA* and *ZPBP2* that were generated in Dr. Laprise's laboratory. I conducted the 5-aza-dC treatment experiments in NuLi-1 and HEK293T/17 cells; extracted DNA and RNA; performed all DNA methylation analyses for HEK293T/17 and analysis of several genes for NuLi-1 experiments using sodium bisulfite sequencing methylation assays; expression and genotyping analysis of the 17q21 region genes and *SLC22A5*.

I analyzed *ZPBP2* DNA methylation in 21 fibroblast cell lines using the sodium bisulfite sequencing methylation assay and analyzed the results. I performed *KDM5C* knock-down experiments using HEK293T/17 cells, i.e. transfected HEK293T/17 with *KDM5C*-specific small interfering RNA (siRNA), performed RNA and DNA extractions and analyzed DNA methylation and expression of 17q21 genes in the transfected cells and controls.

Contributions of the authors in experiments discussed in this thesis are as follows: the Saguenay-Lac-Saint-Jean (SLSJ) asthma familial collection blood samples were provided by Dr. Catherine Laprise (affiliation). Pyrosequencing methylation assays were performed by Dr. Laprise's students and lab members, including Valerie Gagne-Ouellet, Andréanne Morin, Vanessa T. Vaillancourt, and Anne-Marie Madore.

In chapter 2, transfection of HEK293T/17 with siKDM5C, siCTCF and siP300 to confirm the specificity of the siKDM5C construct was conducted by Dr. N. Fotouhi-Ardakani.

In chapter 3, (3.2.8. 5-aza-dC treatment changes DNA methylation at *ZPBP2* and *GSDMA* promoters), several methylation assays in NuLi-1 cells were conducted by S. Moussette and Dr. Hamid-Reza Kohan-Ghadr. The allelic expression assay for *ORMDL3* was done by S. Moussette.

LIST OF MY PUBLICATIONS

1- Berlivet, S., Moussette, S., Ouimet, M., Verlaan, D.J., Koka, V., **Al Tuwaijri, A**., Kwan, T., Sinnett, D., Pastinen, T., and Naumova, A.K. (2012). Interaction between genetic and epigenetic variation defines gene expression patterns at the asthma-associated locus 17q12-q21 in lymphoblastoid cell lines. Human genetics 131, 1161-71.

2- Naumova, A.K., **Al Tuwaijri, A.**, Morin, A., Vaillancourt, V.T., Madore, A.M., Berlivet, S., Kohan-Ghadr, H.R., Moussette, S., and Laprise, C. (2013). Sex- and age-dependent DNA methylation at the 17q12-q21 locus associated with childhood asthma. Human genetics 132, 811-822.

3- **Al Tuwaijri, A.**, Gagne-Ouellet, V., Madore, A.M., Laprise, C., and Naumova, A.K. (2016). Local genotype influences DNA methylation at two asthma-associated regions, 5q31 and 17q21, in a founder effect population. Journal of medical genetics 53, 232-41.

4- Moussette, S., **Al Tuwaijri, A.**, Kohan-Ghadr, H.R., Elzein, S., Farias, R., Berube, J., Ho, B., Laprise, C., Goodyer, C.G., Rousseau, S., et al. (2017). Role of DNA methylation in expression control of the IKZF3-GSDMA region in human epithelial cells. PloS one 12, e0172707. (Shared first authorship).

CHAPTER 1: LITERATURE REVIEW

1.1. DNA methylation

Conrad Waddington introduced the term of epigenetics in the early 1940s, although with time, it has been redefined. The definition of epigenetics is the study of heritable changes of a phenotype, such as gene functional activity of the indicated cell type, that occur independent of changes in the DNA sequence (genotype) (reviewed in (Tollefsbol, 2011, Moore et al., 2013). In a broader sense, epigenetic mechanisms could be responsible for the difference in gene expression levels that has been demonstrated in multiple tissues in eukaryotic cells. DNA methylation represents one of the most widely studied epigenetic mechanisms in most of multicellular organisms. DNA methylation refers to transferring a methyl group from S-adenosylmethionine (SAM) to the fifth position of the pyrimidine ring of cytosine (5-methylcytosine, or 5mC) and occurs in both prokaryotes and eukaryotes. The addition of the methyl group is catalyzed and maintained by DNA methyltransferases (DNMTs) (reviewed in (Moore et al., 2013)).

1.1.1. Location of DNA methylation

In vertebrates, about 72% of annotated gene promoters are located within a CpG island. There are almost 20,000 regions of CGI distributed throughout mammalian genomes (Saxonov et al., 2006) (reviewed in (Vinson and Chatterjee, 2012)). All the housekeeping (HK) gene and some of the tissue-specific gene promoters are embedded in CGIs (Larsen et al., 1992, Gardiner-Garden and Frommer, 1987). HK genes in CpG islands are unmethylated, which suggests that CGI are probably crucial for the regulation of expression of vertebrate HK genes by regulating the chromatin structure and transcription factor binding (Gardiner-Garden and Frommer, 1987). CGI islands, in particular those that reside within promoters and TSS, show high levels of sequence conservation between humans and mice, which suggests that these sites possess a functional importance (Illingworth et al., 2010). DNA is wrapped around protein molecules called histones, which together form nucleosomes. Promoters of active genes with CGI in their TSS usually have nucleosome-depleted regions (NDRs) or nucleosome free regions (reviewed in (Deaton and Bird, 2011)). Methylation of CGI in TSS leads to silencing of gene expression. Another way repression of genes expression is achieved is by polycomb repression proteins (Gal-Yam et al., 2008). DNA methylation of CGI islands plays a pivotal role in regulating gene expression during development

and differentiation, in addition to regulating imprinted genes (reviewed in (Deaton and Bird, 2011)).

Almost half of all CGIs do not reside in annotated TSSs. Some of them are isolated from gene promoter regions and are called ‘‘orphans’’ (Illingworth et al., 2010). Many orphan CGIs involved in transcriptional initiation are frequently methylated during development (Illingworth et al., 2010). Contrary to the CGIs in TSS, CG islands located in intragenic and gene bodies show variation in DNA methylation levels and exhibit tissue-specific methylation patterns (Farthing et al., 2008). An example of a non-CGI TSS is pro-opiomelanocortin (*POMC*), which has CGI located in the 3’ end that initiate transcription in the germline (Gardiner-Garden and Frommer, 1994).

Unlike gene promoter regions, CGIs are not frequently found in gene bodies in the mammalian genome, and the methylation of CpG in gene bodies is positively correlated with the level of gene expression (Jones, 1999). Shann *et al.* studied DNA methylation in human tissues and breast cancer cell lines and found that hypomethylation of tumour suppressor gene bodies is associated with a decrease in the expression of these genes (Shann et al., 2008). Another study used next-generation sequencing technologies to study DNA methylome at the genome scale in human B-lymphocytes, fibroblasts and induced pluripotent stem cells, and they found that hyper-methylation of gene bodies is associated with increased gene expression (Ball et al., 2009). However, it is still unclear how the methylation of the gene body influences gene regulation.

CGI shores, located as far as 2,000 bp away from CGIs, contain low CpG density (Irizarry et al., 2009). Irizarry *et al.* found that more than 16,000 regions of highly conserved, tissue-specific, differentially methylated regions (T-DMRs) in colon cancer tissues, and these T-DMRs located in CGI shores. They showed that the methylation pattern of these CGI shores was strongly associated with gene expression (Irizarry et al., 2009). In agreement with previous finding, Rao *et al.* found that the Caveolin-1 (*CAV1*) oncogene has differentially methylated regions residing in CGI shores but not CGIs as assessed in 30 breast cancer cell lines, and the methylation levels were strongly correlated with *CAV1* expression (Rao et al., 2013). Perisic *et al.* showed that DMR in the glutamate transporter subtype 1 (*GLT-1*), the main astrocytic glutamate transporter, occurred in CGI shores and acted as an enhancer (Perisic et al., 2012).

Enhancers are *cis*-acting regulatory elements that mainly increase the transcription of genes when they bind to transcription factors in a way that is independent of their orientation. They can also function at a distance from their target. Usually, enhancers have a low CpG content (reviewed in (Pennacchio et al., 2013)). It has been suggested that most DMRs located in the enhancers of cell type-specific genes based on DNA methylation analysis performed in different types of human T cells (Schmidl et al., 2009). Aran *et al.* undertook a comprehensive study to determine the relationship between the DNA methylation of enhancers and the dysfunctional regulation of cancer genes in 58 cell types. They found that decreases in DNA methylation levels of enhancers, rather than promoter regions, was associated with upregulation of cancer-related genes, which suggests the importance of enhancer methylation as an indicator of cancer gene expression (Aran et al., 2013). Bell *et al.* conducted a comprehensive DNA methylation and RNA-seq analysis on thousands of patients with diverse cancer severity, and they found that enhancers' differently methylated regions (eDMR) were usually associated with specific metastatic sites, and they suggested that eDMRs are considered the best predictor for cancer progression (Bell et al., 2016).

Insulators play an essential role in the general regulation of distinct gene transcription by blocking the interaction between an enhancer and a promoter (Le Gall et al., 2015). An example of a well-known insulator is the CCCTC-binding factor (CTCF) binding site. DNA methylation at CpG in the CTCF binding site within the imprinted insulin-like growth factor 2 (*IGF2*)–*H19* regions plays an important role in reducing an enhancer's binding affinity and regulating its interaction with the gene promoter (Bell and Felsenfeld, 2000). Shukla *et al.* observed that DNA methylation inhibited the CTCF insulator from binding to exon 5 of the *CD45* gene, which affects regulation of splicing (Shukla et al., 2011).

1.1.2. DNA methyltransferases

There are three members of the mammalian DNA methyltransferase (DNMT) family that are responsible for establishing and maintaining the addition of methyl groups onto DNA: DNA methyltransferases 1 *(*DNMT1*),* DNA methyltransferases 3A and B (DNMT3A and DNMT3B). However, while these enzymes share some structural similarities, each one is functionally different (reviewed in (Kabesch et al., 2010)).

DNMT1 resides in the replication fork where the synthesis of hemimethylated DNA takes place. After DNMT1 binds to the newly synthesized DNA stand, it starts copying the pre-existing methylation pattern of the original strand to the newly synthesized one, which explains the involvement of DNMT1 in maintaining DNA methylation patterns (Hermann et al., 2004).

Although DNMT3A and DNMT3B enzymes are encoded by different genes, they are highly similar structurally, especially in the carboxyl terminal catalytic domain. Both of them belong to the DNMT3 family DNMT3A and DNMT3B are responsible for *de novo* methylation during development, and they do not have a preference for binding to hemimethylated DNA (Okano et al., 1998). Another member of the DNMT3 family is DNMT3L. It shares sequence similarity with *DNMT3A* and *DNMT3B*. However, DNMT3L does not have enzymatic activity due to a loss of the catalytic domain (reviewed in (Moore et al., 2013)). Peptide interaction assays showed that DNMT3L recognizes and binds to sequences of unmethylated lysine 4 on histone H3 tails, which induces *de novo* DNA methylation through interactions with DNMT3A (Ooi et al., 2007).

1.1.3. Function of DNA methylation

Currently, due to major advances in DNA methylation analysis, countless studies have been pursued to garner understanding of the significant role of DNA methylation in different processes, including imprinting, X inactivation, cell differentiation, aging, etc. The role of DNA methylation in selected processes is briefly summarized below.

1.1.3.1.Tissue and cell type-specific DNA methylation

The mammalian genome is characterized by tissue-dependent and differentially methylated regions in which cell type-specific DNA methylation levels play an essential role. The first event of cellular differentiation during mammalian development is the distinguishing of the trophoblast from the embryonic cell lineages (Cross et al., 1994). At embryonic day 3.5 in mice, these two cell lineages give rise to trophectoderm (TE) and inner cell mass (ICM) of blastocysts, in which each cell type has its distinguish DNA methylation pattern (Cross et al., 1994). There are many genes involved in cell type- and tissue-specific methylation. Transcription factor E74-like factor 5 (*ELF5*) is essential for the maintenance of trophoblast stem cells (TSC), which it tissue-dependent and differentially methylated region (T-DMR) shows a hypomethylation status in TSC and can be

repressed by highly methylated loci in embryonic stem cells (ESC) (Ng et al., 2008). Conversely, The *Nanog* and *Oct-4* T-DMRs are highly methylated in TSC and hypomethylated in ESC (Hattori et al., 2004, Hattori et al., 2007). Mouse ESCs lack Dnmt1, and *de novo* methyltransferases Dnmt3a and Dnmt3b maintain their self-renewal ability but lose the cells' ability to differentiate (Tsumura et al., 2006).

1.1.3.2. Age-dependent DNA methylation

Aging is a cumulative process, and the gradual deterioration of the molecular and cellular structures that make up an individual are affected by environmental changes that can reduce the probability of survival (Schumacher, 2011). Signs of epigenetic modifications, such as DNA methylation, have been associated with aging (Schumacher, 2011). Bollati *et al.* showed that there was a decrease in the average DNA methylation state of arthrobacter luteus (*Alu*) elements in elderly individuals between 55 and 92 years of age. The DNA methylation state was assessed throughout an 8-year timespan, and the reduction in Alu methylation levels was highly correlated with time since the first assessment (Bollati et al., 2009). It has been shown that DNA methylation is significantly associated with age based on a study of methylation profiles conducted in over 200 non-pathologic human DNA samples from 10 different tissues using pyrosequencing technologies. In this study, they found that in solid tissues, genomic regions located in CGIs gained more methylation with age, while there was a significant lose of DNA methylation in loci located outside of CGIs (Christensen et al., 2009). There are several genes whose methylation is affected by aging, such as cellular myelocytomatosis (*c-Myc*). In aged mice, *c-Myc* showed high DNA methylation levels in the liver and hypomethylation in the spleen, whereas it showed no changes in brain at the same time (Ono et al., 1986).

1.1.3.3. Environmental effect on DNA methylation

Global and gene-specific DNA methylation changes have been observed in most of the studies conducted on epigenetic modifications induced by environmental factors (reviewed in (Arita and Costa, 2011)). The factors that influence DNA methylation include dietary methyl donors, tobacco smoke, and farm exposure, which affects the susceptibility of developing chronic diseases by epigenetic mechanisms, (reviewed in (Arita and Costa, 2011)). Selected examples are briefly summarized below.

One of the best models that illustrates how environment affects DNA methylation is the viable yellow agouti mouse. In these mice, the coat color is indicated by the expression of the agouti gene. However, transposable element insertion of an intracisternal A particle (IAP) located in a regulatory region of the agouti locus leads to a different form of the agouti viable yellow (Avy) allele (Duhl et al., 1994). The offspring of mothers fed with a diet rich in methyl donors and cofactors showed changes in coat color, which becomes like the wild type coat color when compared with the offspring of mothers fed on the normal diet (Waterland and Jirtle, 2003). The hypermethylation of IAP caused the mice's coat color to change from yellow to the brown agouti coat color, identical to that of wild-type mice (Waterland and Jirtle, 2003). Also, feeding methyl donor supplementation to the axin fused (AxinFu) female mice before and during pregnancy caused a remarkable decrease in the incidence of tail kinking in the offspring due to hypermethylation of IAP retrotransposon into axin (Waterland et al., 2006).

Buro-Auriemma *et al.* conducted a genome-wide DNA methylation analysis to describe the effects of active smoking on the small airway epithelium methylome in smoker and non-smoker subjects, and found several changes in DNA methylation with corresponding modulation of gene expression (Buro-Auriemma et al., 2013). Cigarette smoke increases the methylation level of the tumor suppressor gene, Nischarin (*NISCH*), in the plasma of heavy smokers and individuals with lung tumors (Ostrow et al., 2013).

1.1.3.4. Genotype-dependent effects on DNA methylation

It has been shown that genetic variation contributes to inter-individual variation in DNA methylation levels. Bell *et al.* studied DNA promoter methylation across the genomes of 77 HapMap lymphoblastoid cell lines (LCLs). They observed significant negative correlation between DNA methylation and gene expression. They found that methylation levels mostly correlated across a 1-2 kb genomic interval, and they also noticed when pairs of CpGs were both located in the same CGI, they showed similar methylation patterns compared to pairs of CpGs where one was located outside the CGI (Bell et al., 2011). They detected genome-wide association of DNA methylation with SNPs in *trans*. They also revealed cis-association between SNPs and genes located at considerable distances (e.g. 165 kb for the methyl-binding protein 2 (*MBD2*); 22 kb from *DNMT1*; and 192 kb from *DNMT3B*) (Bell et al., 2011). Dayeh *et al.* found 19 out of 40 SNPs introduce or abolish a CpG site and were associated with type 2 diabetes. DNA methylation

of these CpG-SNPs was assessed in islets of non-diabetic human donors. The association between the type 2 diabetes CpG-SNPs and differential DNA methylation of the CpG-SNP site in human islets was found. Some of these CpG-SNPs influenced gene expression, while others associated with differential DNA methylation at proximal CpG sites (Dayeh et al., 2013). Furthermore, in a chromosome-wide survey of allele-specific DNA methylation (ASM) study that was conducted in 16 human pluripotent and adult cell lines, they noticed about 30% of heterozygous SNPs showed local ASM, and most of ASM are part of CpG-SNPs, which affect methylation levels. They suggested that CpG-SNPs act as cis-regulatory polymorphisms that connect genetic variation to interindividual differences in DNA methylation (Shoemaker et al., 2010). Another study assessed the DNA methylation of prodynorphin (*PDYN*) CpG-SNPs associated with alcohol dependence in postmortem prefrontal cortex samples from alcohol-dependent individuals and controls and found three differently methylated CpG-SNPs associated with alcoholism. The non-risk allele of the *PDYN* is methylated in alcohol-dependent samples (Taqi et al., 2011). Reversion-induced LIM gene (*RIL*) is a tumor suppressor gene that is repressed by hypermethylation in the tissues of patients with prostate cancer (Vanaja et al., 2006) deleted in myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) patients (Boumber et al., 2008). Boumber *et al*. (2008) demonstrated that *RIL* has short and long alleles. The long allele has a 12 bp polymorphic region located near its transcription start site. In leukemia and colon cancer, the long allele showed decrease in DNA methylation levels than the short allele, and the long allele creates a specificity protein 1 and 3 (SP1/SP3) binding site that showed a protective mechanism against methylation in cancer (Boumber et al., 2008).

1.2. Asthma

1.2.1. Asthma characterization and classification

Asthma is one of the common chronic diseases that affect people of all ages. It is a complex genetic disease with a heterogeneous phenotype, in which multiple genes and environmental factors play a role in pathogenesis (Zhang et al., 2012). Asthma is characterized by intermittent inflammation and airway remodeling, which cause airway hyperresponsiveness (AHR) and airway obstruction (Cohn et al., 2004). Asthma shows a spectrum of disease phenotypes, and clinical symptoms include small airway inflammation, wheezing, chest tightness, dyspnea and shortness of breath. These symptoms present in variable levels of severity and frequency from patient to

patient (Kumar and Ghosh, 2009, Ober and Hoffjan, 2006, Zhang et al., 2012). Asthma is classified into two distinct phenotypes: extrinsic (allergic) or intrinsic (non-allergic) asthma. Both phenotypes involve eosinophilic inflammation (Eng and DeFelice, 2016, Agache et al., 2012). However, extrinsic asthma is triggered by airborne, inhaled allergens such as pollen, which lead to hypersensitivity reactions mediated by increased serum Immunoglobulin E (IgE), inducing bronchial hyperresponsiveness. It typically affects children and is more common in boys. Extrinsic asthma is generally more responsive to treatment compared to non-allergic asthma, which is considered to be driven by factors unrelated to the immune system. Intrinsic asthma is not believed to be triggered by any specific allergens. It usually affects adults, more commonly women than men, and it is difficult to treat (Eng and DeFelice, 2016, Kumar and Ghosh, 2009, Agache et al., 2012).

1.2.2. Childhood asthma

Childhood asthma is one of the most common chronic disease among children, with a high prevalence in many countries. Over seven years (1994-2001), the rate of Canadian children aged 0 to 11 diagnosed with asthma was estimated to have increased from 11% to 13%, translating to roughly 70,000 new cases (Garner and Kohen, 2008). In Quebec, the public healthcare cost associated with pediatric asthma during the years 1994-95, including medical/nursing care, medication and equipment use, was estimated to total $11 million, representing a significant burden for the economy (Bahadori et al., 2009). Childhood asthma occurs more frequently in younger boys than girls before puberty, although the ratio shifts in adulthood, with a higher prevalence of asthma in women than men (Garner and Kohen, 2008, Almqvist et al., 2008). In early life, viral infections and recurrent wheezing due to airway obstruction are the main symptoms of childhood asthma. An exaggerated inflammatory response is exacerbated by intermittent viral infections (Garner and Kohen, 2008).

1.2.3. Inflammatory and immune cells involved in asthma

Generally, asthmatic individuals have high levels of mast cells, basophils, lymphocytes, macrophages and eosinophils (Kumar and Ghosh, 2009, Szefler et al., 2014). Mast cells play a contributory role in asthma by secreting many bronchoconstrictors such as histamine, leukotrienes and prostaglandin. Also, they are responsible for the release of cytokines that are associated with

allergic inflammation, including interleukin 4 (IL4), IL5 and IL13. The infiltration of mast cells into the smooth muscles of the airway has been demonstrated in asthmatic patients with airway hyperresponsiveness (reviewed in (Morita et al., 2016)). The airway of asthmatic patient also has increased numbers of CD4+ T cells, which are mostly T helper 2 (TH2) cells, whereas in a healthy, unaffected airway, T helper 1 (TH1) cells predominate. Their main role is releasing the cytokines IL4 and IL13, which influence the secretion of IgE by B cells, as well as IL5 and IL9, which are responsible for eosinophil and mast cell differentiation, respectively. CD4+ T cells have different subsets, including regulatory T cells, that influence the function of TH2 cells in asthma; TH17 cells, which secret IL-17 that is found predominantly in the sputum of asthma patients; and invariant natural killer T (*i*NKT) cells, which are responsible for IL4 and IL13 secretion. It has been reported that about 60% of all CD4+ T cells found in bronchial biopsies from asthmatic patients were *i*NKT (Cosmi et al., 2011) (reviewed in (Jutel and Akdis, 2011, Barnes, 2008)). In childhood asthma, eosinophilia is observed on bronchoalveolar lavage in older children but is less frequently observed in infants. Despite these common cell types, some asthmatic children may present a non-eosinophilic or neutrophilic type of asthma. Most asthmatic children manifest an airway remodeling phenotype, which involves epithelial cell injury and thickening of a variety of airway tissues, including smooth muscle hypertrophy, hyperplasia, and angiogenesis. These changes lead to airflow limitation and increased airway hyperresponsiveness, which further exacerbates symptoms (Szefler et al., 2014, Kabesch, 2016).

1.2.4. The role of environmental factors in childhood asthma

Asthma onset has a strong environmental component. The hygiene hypothesis was proposed by Strachan in the late 1980s. Based on the inverse relationship between family size and children's predisposition towards allergies, the hypothesis states that the cause of the rapid increase in the prevalence of asthma and atopic disorders is the lack of childhood exposure to infectious agents (Strachan, 1989). This hypothesis was supported by Olszak et al. who found that there was an accumulation of iNKT cells in the lungs of mice raised under sterile, germ-free conditions and an increase in death due to allergic asthma (Olszak et al., 2012). Moreover, Varraso et al. confirmed the farming/hygiene hypothesis and found that contact with livestock early in life and a farming lifestyle protect against children developing asthma (Varraso et al., 2012). Recently, a comprehensive, systematic review and meta-analysis used published studies from 1960 until

November 2013 to demonstrate an association between psychological stress during pregnancy and an increased prevalence of childhood asthma (van de Loo et al., 2016). It has been indicated that exposure to respiratory viral infections such as respiratory syncytial virus and human rhinovirus (HRV) in early life increases the risk of asthma development (Jackson et al., 2008, Krishnamoorthy et al., 2012). Other environmental factors associated with increased asthma development during childhood include tobacco smoke, air pollution and antibiotics (Burke et al., 2012, Gasana et al., 2012, Marra et al., 2006).

1.2.5. The genetic component of asthma

Screening the genome for asthma susceptibility loci began during the past few decades, and since then, significant advances have been achieved in the field of genetics. Through this research, several pathways that are implicated in asthma pathogenesis have been unveiled, showing that asthma and asthma-related traits act as complex diseases (reviewed in (Vercelli, 2008)). However, there are still several challenges that need to be resolved. Asthma is a polygenic, multifactorial disease. Not all the genes involved in its onset and progression have been identified yet. Identifying a common mechanism underlying the wide range of asthma phenotypes is also challenging given the heterogeneity of the condition across patients. It has been challenging to replicate genotype-phenotype associations due to variation among populations. Finally, understanding how environmental factors interact with genetic and epigenetic factors to influence asthma susceptibility also remains elusive (reviewed in (Vercelli, 2008)). The contribution of genetics in asthma pathogenesis has been demonstrated in several twin studies, with overall heritability estimates ranging from 35% to 80% (Thomsen et al., 2010, Murphy et al., 2015).

1.2.6. Genome-wide association studies (GWAS) of asthma

The completion of the Human Genome Project in 2003 and the International HapMap Project in 2005 has facilitated the genomic research to unlock the complexity of asthma and enhance our understanding of other complex human diseases. Studies of the genetics of asthma have been conducted using several approaches, one of which involves genome-wide association studies (GWAS). GWAS involve a hypothesis-independent, powerful approach used for mapping genetic risk loci for common complex diseases by analysing the entire genome. These studies often involve identifying associations between common genetic variants, often referred to as ‘markers,’

that have no less than 1% minor allele frequency (MAF) in the population with the disease phenotype (Bush and Moore, 2012). The number of analysed markers in GWAS has reached more than one million single nucleotide polymorphisms (SNPs) (Zheng et al., 2012). The main advantages of GWAS lie in their ability to uncover novel candidate gene variants, mainly those with moderate risks, while also providing an unbiased approach to identifying SNPs without relying on the previously established knowledge surrounding disease etiology. However, as with any other method, there are some limitations. Patient recruitment for such studies can be a burden and demands a large sample size to reach genome-wide levels of significance (within $P < 10^{-7}$). Genetic heterogeneity in many samples raises the problem of false positive results that cause challenges in bioinformatics (Stranger et al., 2011) (reviewed in (Ober and Yao, 2011)). Recently, more than 100 GWAS loci located on autosomal and sex chromosomes were associated with asthma and asthma-related traits, but none of them alone explains even a small percentage of asthma phenotypes (Lee et al., 2015). Examples from the best-replicated childhood asthma-associated genes based on GWAS results are summarized in (Table 1.1).

1.2.7. Sex differences in the genetic association for asthma risk

Sex differences in the genetic risk association for asthma have been well described for some asthma-associated regions, although the genetic or/and epigenetic causes that may explain this bias have not received enough attention (Osman, 2003). Asthma is a sexually dimorphic disease that is influenced by age. The sex ratio in childhood asthma is significantly biased towards males, whereas it is reversed during and after puberty (Osman, 2003). Moreover, several studies showed sex differences in immune response profiles related to the development of atopic disease and childhood asthma. For example, three-year-old asthmatic boys show elevated IL5 and IL13 cytokine responses and increased total IgE levels and eosinophil counts compared to girls (Uekert et al., 2006). Loisel *et al.* studied genotype-by-sex interactions in children at high risk for asthma and allergic diseases (Loisel et al., 2011). Males that were heterozygous for interferon gamma (*IFNγ*) alleles showed higher risk of developing asthma compared to heterozygous females. The interactions between genotype and sex were also found in the *IFNγ* response to lipopolysaccharides (LPS) in cord blood samples (Loisel et al., 2011). In another study, two SNPs located in the thymic stromal lymphopoietin (*TSLP*) locus were significantly associated with asthma. One of them was associated with a lower risk of developing asthma in males while the other was exclusively

associated with a reduced risk of asthma in females (Hunninghake et al., 2010). Moreover, similar findings have been reported in a sex-stratified linkage analysis study conducted in 295 families to characterize asthma-related phenotypes, including IgE levels, allergen polysensitization, eosinophil counts and lung function forced expiratory volume 1 (FEV_1)/height^2. A statistically significant, male-specific association was found between *IL9* located on chromosome 5q31 and lung function and allergen sensitization (Aschard et al., 2009). A recent genome-wide meta-analysis of association studies of a pool of over 5,000 asthmatic individuals and 3,830 non-asthmatic controls found a statistically significant association for asthma in European and American males only at SNPs close to interferon regulatory factor 1 **(***IRF1*) in the 5q31 asthmatic region (Myers et al., 2014). A transmission disequilibrium test (TDT) was conducted in 1,214 samples distributed in 240 families of the Saguenay Lac-Saint-Jean familial collection (SLSJ), which indicated a strong genetic association between asthma and two SNPs: rs9303277 and rs4795405 in the 17q21 chromosomal region among males (Naumova et al., 2013).

1.3. The chromosomal region 5q31 associated with asthma

Studies of asthma genetics have pointed to genetic regions that contribute to the control of immunoglobulin E (IgE) levels. Epidemiological studies have indicated an interaction between elevated IgE levels and an increased risk of developing asthma (Burrows et al., 1989). The 5q31 region was investigated by several linkage analysis studies, as it includes a cluster of cytokine genes which play an important role in the pathogenesis of asthma and atopy. A linkage analysis study was conducted in a large group of Caucasian-Amish families. The linkage between total serum IgE levels and several genetic markers in 5q31 were tested, and they proposed that one or several functional genetic variations existing within the *IL4* gene in the 5q31 loci was responsible for regulation of IgE secretion (Marsh et al., 1994). Another linkage analysis study was performed on families from Northern Holland. They found evidence of linkage between IgE serum levels and the 5q31 region, and they suggested that there were multiple candidate loci in 5q31 associated with the asthma phenotype, especially given that elevated total serum IgE levels were correlated to a clinical diagnosis of asthma (Meyers et al., 1994). The linkage between the 5q31 region and increased IgE levels was confirmed and has since been replicated in ethnically diverse populations. However, none of these studies has sufficient statistical power to accurately predict the complete region of candidate gene(s) (Postma et al., 1995, Xu et al., 1995, Doull et al., 1996). Eosinophils

are one of the most common inflammatory cell types seen in the bronchi of asthmatic patients. Hence, GWAS were conducted on DNA from blood eosinophils from over 9,000 Icelandic individuals to determine whether genes associated with eosinophil levels are also associated with developing asthma (Gudbjartsson et al., 2009). The number of investigated SNPs was narrowed down and the study was replicated in another Icelandic cohort, the SNPs with the smallest *P* values were re-investigated in a large sample of asthmatic patients from Europe and East Asia. In this larger cohort, they found a significant association between the rs2416257 SNP in WD Repeat Domain 36 (*WDR36*) in the 5q22.1 region and both blood eosinophil levels and asthma ($p = 4.2\times10^{-6}$) (Gudbjartsson et al., 2009). Another large-scale collaborative GWAS investigated SNP associations in over 10,000 asthmatic patients and more than 16,000 controls. This study found an association between asthma and the rs2073643 SNP near solute carrier family 22 member 5 (*SLC22A5*) in the 5q31 region ($p = 2\times10^{-7}$) (Moffatt et al., 2010). After that, the association between the 5q31 region and asthma has been confirmed in several polymorphisms in 5q31 and replicated in several other population studies (Li et al., 2010, Laprise, 2014). Common SNPs in the 5q31 region are also associated with other autoimmune diseases, such as Crohn's disease, which suggests that asthma and Crohn's disease may have shared mechanisms that are still under investigation (Figure 1.1) (Kenny et al., 2012, Liu et al., 2015, Jostins et al., 2012). The 5q31 region show some of the highest genome-wide significance scores for allelic expression differences in 53 LCLs suggesting genetic variants in regulatory elements may acting in cis (Ge et al., 2009). They found significant differences in allelic expression on *SLC22A5* RNA, and mapped it to a genomic interval of about 240-kb in 5q31 region encompassing (organic cation/zwitterion transporter), member 4 (*SLC22A4*), *SLC22A5* and *IRF1* (Ge et al., 2009). They also, indicated a suggestive allelic expression differences for PDZ and LIM domain 4 (*PDLIM4*), *SLC22A4* and *IRF1*(Ge et al., 2009). Aligning this data with data from GWAS (Franke et al., 2010, Moffatt et al., 2010, Torgerson et al., 2011, Kenny et al., 2012) we determined the alleles that were associated with higher expression of *SLC22A5* were also associated with predisposition to asthma and we named it haplotype C (HapC alleles), and the alleles that were associated with lower *SLC22A5* expression were not associated with predisposition to asthma haplotype D (HapD alleles). The genomic region that was investigated by our laboratory located at 240-kb region in 5q31 that spanned several loci, including the following genes: interleukin 3 (*IL3*), colony-stimulating factor 2 (*CSF2*), prolyl 4-hydroxylase, alpha polypeptide II (*P4HA2*), PDZ and LIM domain 4

(*PDLIM4*), solute carrier family 22 (organic cation/zwitterion transporter), member 4 (*SLC22A4*), *SLC22A5,* interferon regulatory factor 1 (*IRF1*), and interleukin 5 (*IL5)* (Al Tuwaijri et al., 2016).

1.3.1. Solute carrier family 22 (organic cation/ carnitine transporter), member 5 (*SLC22A5*)

SLC22A5 or (OCTN2) is part of a larger family named organic cation transporter. *SLC22A5* is a Na^+-dependent plasma membrane cation transporter that has a major role in transferring carnitine through the cell membrane into the mitochondria (reviewed in (Pochini et al., 2013)). Carnitine is a water soluble amino acid that plays an essential role in long-chain fatty acid transportation through the mitochondrial matrix, which contributes in the oxidation of glucose and production of cellular energy (reviewed in (Mongioi et al., 2016)). *SLC22A5* is expressed in several tissues, including the intestines, kidneys, placenta, heart, testis, skeletal muscles, and brain (reviewed in (Pochini et al., 2013)). Several studies conducted in ethnically diverse populations have indicted the association between mutations in *SLC22A5* and Crohn's disease development (Peltekova et al., 2004, Yamazaki et al., 2004). Shekhawat *et. al* studied the influence of carnitine deficiency in the gastrointestinal tract of homozygous *Slc22a5*$^{-/-}$ null mice. They noticed that the mice suffered from abnormal villi structures, inflammation, lymphocytic and macrophage infiltration, significant apoptosis in gut epithelial cells, and atrophy of the small intestine and colon. Furthermore, carnitine deficiency predominantly affected birth weight. Mice at three weeks of age had half the body weight of their wild type counterparts (Shekhawat et al., 2007). Another study was done by the same group they investigated the neonatal gut of homozygous-deficient mice (*Slc22a5*$^{-/-}$). It showed that deficiency of carnitine influenced the immune system and caused severe atrophy and apoptosis in splenocytes, thymocytes, and lymph node lymphocytes (Sonne et al., 2012). It has been indicated that mutations in *SLC22A5* lead to loss of OCTN2 carnitine transporter function, which causes primary systemic carnitine deficiency (PSCD). The expression of *SLC22A5* has been indicated in human and mouse kidneys, which suggests the possible role of *SLC22A5* in the reabsorption of carnitine from urine to maintain carnitine homeostasis in the plasma (Nezu et al., 1999). During intestinal inflammation, there is an increase in the production of pro-inflammatory cytokines such IFNγ and tumor necrosis factor-alpha (TNFα). This increase in secretions then influences the characteristics of the intestinal epithelial cells. *SLC22A5* is predominantly expressed in the apical and subapical regions of the large and small intestinal

epithelial tissue (Fujiya et al., 2011). The expression of *SLC22A5* was upregulated in inflamed colonic tissue from Crohn's disease patients, probably due to increased levels of IFNγ and TNFα cytokines. This suggests the role of *SLC22A5* to be one involving the maintenance of intestinal carnitine levels during inflammation (Fujiya et al., 2011). GWAS in asthmatic and non-asthmatic samples confirmed the association between *SLC22A5* and asthma (Moffatt et al., 2010). *SLC22A5* expressed in the testes, especially in the luminal epithelium, and involved in the motility of the spermatozoa and the maturation of the epididymis (Kobayashi et al., 2005). Expression Quantitative trait loci (eQTL) studies were used to examine RNA that was extracted from postsurgical iliac biopsy specimens, which showed over 15,000 *cis*- and trans-eQTLs with a tissue-specific effect, including rs1050152/*SLC22A4* and *SLC22A5* SNPs, which were associated to inflammatory bowel disease (IBD) (Kabakchiev and Silverberg, 2013).

1.4. The chromosomal region 17q21 associated with asthma

In 2007, Moffatt *et al.* were the pioneers who studied about 317,000 SNPs in DNA samples from 994 asthmatic children and 1,243 non-asthmatic controls using European family and case-referent panels (Moffatt et al., 2007). All samples were genotyped with the Illumina Sentrix HumanHap300 BeadChip. They found multiple SNPs at the chromosome 17q21 locus, which showed a strong association with childhood asthma. They narrowed down the associated region to a 112 kilobase pair (kb) interval on chromosome 17q21. The study was replicated in two independent cohorts. Moreover, they assessed the transcript levels of genes in Epstein–Barr virus (EBV) transformed LCLs derived from asthmatic children and found an association between the expression level of the orsomucoid-like 3 (*ORMDL3*) gene in the 17q21 region and the SNPs associated with childhood asthma (Moffatt et al., 2007). In 2010, Moffatt *et al.* carried out another association study in the 17q21 region using an increased sample size of 10,365 asthma patients (asthmatic adults were included alongside asthmatic children) and 16,100 unaffected controls. In this study, more than 500,000 SNPs were genotyped. The same previously identified region on chromosome 17q21 that showed association with childhood asthma was expanded to include an approximately 380-kb region of implicated loci. They found SNPs in gasdermin B (*GSDMB*) and gasdermin A (*GSDMA*) in the 17q21 region, which were also associated with childhood asthma. They concluded that genetic polymorphisms in the 17q21 region are exclusively associated with childhood asthma and not adult asthma (Figure 1.2) (Moffatt et al., 2010). Another recent

association study conducted in British cohorts also found that the genetic variants in 17q21 do not influence the risk of asthma in adults (Kreiner-Moller et al., 2015). However, there are other studies that have suggested there may be an association between asthma and the 17q21 alleles in adults (Sleiman et al., 2008, Galanter et al., 2008, Hrdlickova and Holla, 2011). Association between the 17q21 region and asthma has been replicated across ethnically diverse populations, including European, French Canadian, Latino, African American, North American, Japanese and Chinese cohorts (Halapi et al., 2010, Madore et al., 2008, Galanter et al., 2008, Sleiman et al., 2008, Torgerson et al., 2011, Hirota et al., 2008, Leung et al., 2009, Yang et al., 2012). Besides childhood asthma, other autoimmune diseases such as Crohn disease, ulcerative colitis, Type 1 diabetes, biliary cirrhosis and rheumatoid arthritis are all associated with the same genomic region in 17q21 (Barrett et al., 2008, Barrett et al., 2009, Stahl et al., 2010, Liu et al., 2010b, Anderson et al., 2011). Therefore, understanding the underlying mechanisms regulating the 17q21 region can play an important role in improving our understanding of the pathogenesis of complex diseases.

1.4.1. 17q21 genes

A genetic association study mapped the cis-regulatory haplotype that leads to allele-specific differences in gene expression in the LCLs, CD4+ and CD8+ T-lymphocytes in centre d'etude du polymorphisme humain (CEPH) (Utah Residents having Northern and Western European Ancestry) (CEU). This study mapped a 160-kb region that encompasses the IKAROS family zinc finger 3 *(IKZF3), ZPBP2, GSDMB* and *ORMDL3* genes. Also, indicated two of the most common cis-regulatory haplotypes: haplotype A (HapA) carries asthma-associated risk alleles and haplotype B (HapB) (sum of all non-HapA haplotypes) that not carries asthma-associated risk alleles. HapA was associated with increased expression of *ORMDL3* and *GSDMB* while *ZPBP2* expression level was decreased. Conversely, the expression of *ORMDL3* and *GSDMB* in HapB was decreased, while *ZPBP2* expression level was increased (Verlaan et al., 2009).

1.4.1.1. Orsomucoid-like 3 (*ORMDL3*)

The *ORMDL3* gene in 17q21 region received most of the attention and was extensively and repeatedly studied in ethnically diverse populations. Most of the association studies linked *ORMDL3* to asthma. Hence, several studies consider it to be a candidate gene for causing a

predisposition towards childhood asthma. *ORMDL3* belongs to an *ORMDL* gene family containing three genes in humans (*ORMDL1*, *ORMDL2* and *ORMDL3*) (Hjelmqvist et al., 2002) (reviewed in (Paulenda and Draber, 2016)). ORMDL proteins localize predominantly in the endoplasmic reticulum (ER) and are highly conserved between species (Hjelmqvist et al., 2002). These proteins are also extensively expressed in fetal and adult mammalian tissues that involve lung epithelial cells (Miller et al., 2012). There is 80% similarity between ORMDL amino acids in three human paralogs, whereas the similarity between human and mouse orthologs is 95% (Hjelmqvist et al., 2002).

1.4.1.2. Gasdermin A (*GSDMA*)

A member of the gasdermin family is *GSDMA*. A study of *GSDM* gene family expression levels in normal and tumour gastrointestinal epithelium cells revealed that *GSDMA* is involved in cell growth inhibition activity through apoptosis, and it was found to be expressed in the esophageal and gastric epithelia as well. *GSDMA* acts as a tumor suppressor in esophageal squamous cell carcinomas (Saeki et al., 2009). Another study investigated the association of two SNPs located in *GSDMA* and *GSDMB* with asthma in Korean children. They found both variants were associated with elevated total IgE and increased BHR to methacholine, and the variants were also associated with asthma in children (Yu et al., 2011). Furthermore, study used cord blood mononuclear cells and showed that risk variants for asthma in the 17q21 locus were associated with increased *ORMDL3* and *GSDMA* gene expression levels and increased IL17 secretion, which suggests the functional role of the 17q21 region is the regulation of T cells, especially in early life and development (Lluis et al., 2011). Hence, *GSDMA* cannot be excluded as a candidate gene for asthma risk in the 17q21 locus (Yu et al., 2011).

1.4.1.3. *Zona pellucida* binding protein 2 (*ZPBP2*)

The *ZPBP2* gene is critical for fertilization and encodes a protein involved in the binding of sperm to the zona pellucida, which surrounds the oocyte. *ZPBP2* is highly expressed in testis and is conserved in mammals (Lin et al., 2007). Genetic analyses mapped a cis-regulatory haplotype of 160-kb in the 17q21 region that was found to cause allelic differences in expression levels in LCLs. A SNP (rs12936231) located in the intronic region of the *ZPBP2* gene affects the binding site of a CCCTC-binding factor (CTCF) and modifies nucleosome occupancy. The

asthma-associated C allele of rs12936231 removes a CTCF-binding site. This binding site is present in non-asthma-associated G allele of rs12936231. CTCF functions as the main insulator protein in vertebrates. When it interacts to insulate DNA elements, it may prevent enhancers from activating target genomic regions. This is achieved through the formation of chromatin loops. Losing the CTCF binding site is associated with upregulation of expression levels of *ZPBP2* (Verlaan et al., 2009). Another study focused on the interaction between genetics and epigenetics in the 17q21 locus in the promoter regions of *ZPBP2*, *GSDMB* and *ORMDL3*. The expression levels of these genes was found to be influenced by the *cis*-regulatory haplotype. They found functional SNP rs4795397 located in intron five of *ZPBP2* showed association with the active histone mark histone H3 lysine 9 acetylation (H3K9Ac) *in vitro,* and it possibly functions as an enhancer that influences the expression of neighboring genes. Moreover, they found that there is a negative correlation between DNA methylation of *ZPBP2* promoter region and expression levels in LCLs (Berlivet et al., 2012). Dimas *et al.*, who searched for sex-specific differences in the expression of quantitative trait loci (eQTLs) in LCLs separately in females and males, showed sex-biased differences in the regulatory effect of the *ZPBP2* gene (Dimas et al., 2012). In exome analysis of children with pediatric inflammatory bowel disease (PIBD) and controls, they found *ZPBP2* nonsynonymous variants in children who have IBD and asthma (Andreoletti et al., 2015). Many lines of evidence will be discussed in the next chapters, and research into the regulatory polymorphisms harbored in the *ZPBP2* promoter region will be presented as well as how the interaction between genetics and epigenetics in this region could influence childhood asthma predisposition.

1.4.2. Association between genetic variants in chromosomal region 17q21 and wheezing phenotype in children

Several studies have reported an association of SNPs in the 17q21 region and childhood wheezing phenotypes. Bisgaard *et al.* found that variations in the 17q21 locus were associated with a two-fold increased risk of recurrent wheezing and asthma exacerbations. However, these effects were restricted to early-onset childhood asthma before the age of three (Bisgaard et al., 2009). Another study found a significant association of three genetic variants in 17q21 locus with childhood wheezing. These SNPs were significantly associated with the transcript levels of *ORMDL3*, *GSDMB* and *IKZF3* (Granell et al., 2013). In both studies, there was no association

between variations in the 17q21 region and intermediate phenotypes such as atopy (Granell et al., 2013, Bisgaard et al., 2009). It has been shown that the association between 17q21 polymorphisms and early-onset asthma is only found in children who have had HRV and not respiratory syncytial viral (RSV) wheezing disease. Additionally, there was a significant upregulation in *ORMDL3* and *GSDMB* expression levels in peripheral blood mononuclear cells (PBMCs) stimulated by HRV as compared with unstimulated ones in a genotype-specific manner (Caliskan et al., 2013).

1.4.3. Environmental triggers influence the association between 17q21 alleles and asthma

Gene-environment interactions play a pivotal role in the development of asthma; therefore, several studies have focused in the relationship between genetic predisposition and exposure to environmental factors. Bouzigon *et al.* found in children who in early life were exposed to tobacco smoke had higher risk of developing early-onset asthma, the risk increases further specially among those carrying the 17q21 asthma-associated risk allele (Bouzigon et al., 2008). Other studies done in North Americans of European ancestry supported Bouzigon *et al.'s* finding that 17q21 polymorphisms interact with exposure to tobacco smoke to contribute to increased risk of developing pediatric asthma. However, they found that variations in the 17q21 region are associated with both early- and late-onset asthma (Flory et al., 2009). Exposure to animal shedding and furry pets protects against the risk of developing a wheeze among carriers of the 17q21 asthma risk allele (Loss et al., 2016, Brauner et al., 2012). In a study on the peripheral blood samples of rural farmers' children, the effect of environment on the changing of DNA methylation patterns in asthma-associated genes was investigated, and a significant change was observed in the DNA methylation levels of asthma-related genes such as the *ORMDL* family as well as IgE regulation of genes such as *RAD50*, *IL13* and *IL4* (Michel et al., 2013). Thus, epigenetics could be one of the underlying mechanisms that influence the gene-environment interaction. Several other studies on DNA methylation have been documented on subphenotypes, triggers and genes involved in predisposing individuals to developing asthma (reviewed in (Lee et al., 2015)).

1.4.4. The epigenetics of asthma

To explore whether variations in DNA methylation levels in early-onset asthma are responsible for the asthma phenotypes, the genome-wide patterns of DNA methylation in buccal

cell samples were analyzed in 37 pairs of monozygotic twins (MZ) using the Infinium Human-Methylation450 BeadChip array. Interestingly, they identified several differently methylated positions (DMP) between MZ twins that have previously been linked to asthma, such as the heparan-α-glucosaminide N-acetyltransferase (*HGSNAT*) gene, which was indicated previously to be down-regulated in atopic asthmatic children (Murphy et al., 2015). Another study used purified Regulatory T cell (Treg) and effector T cell (Teff) DNA from the peripheral whole blood of MZ twins to analyze the transcript and DNA methylation levels of Forkhead box P3 (*FOXP3*) and *IFNγ*. These two genes play a pivotal role in Treg and Teff function and development, and impairment of these cells has been found in asthmatic individuals. They found increased in DNA methylation levels and downregulation of *FOXP3* expression in Treg of asthmatic MZ twins. Moreover, Teff from asthmatic MZ twins showed increased *IFNγ* DNA methylation and decreased *IFNγ* transcript levels when compared to non-asthmatic MZ twins (Runyon et al., 2012).

1.4.4.1. Effect of smoking on DNA methylation in patients with asthma

One of the essential and consistent environmental risk factors that trigger adult- and pediatric-onset asthma as described in several epidemiological studies is tobacco smoke exposure. Breton *et al.* proposed that *in utero*, tobacco smoke exposure can cause a lifelong modification in DNA methylation. They studied the global and promoter CGIs methylation in buccal cells of children exposed to tobacco smoke. They found decreased in methylation levels of AluYb8 repetitive elements, while there were no significant changes for *LINE-1* methylation levels compared to unexposed children (Breton et al., 2009). Recently, new evidence has suggested an important role for DNA methylation in explaining the relationship between *in utero* tobacco smoke exposure and asthma. DNA methylation levels have been analyzed in the whole blood of 527 asthmatic children with prenatal smoking history, and increases in DNA methylation have been indicated in two loci: FERM Domain Containing 4A (*FRMD4A*), which plays a role in membrane trafficking, and chromosome 11 open reading frame 52 (*CLLORF52*) in asthmatic children. The results were also replicated in two additional independent populations (Breton et al., 2014). Differences in DNA methylation have been associated with the development of asthma in adult smokers as well. The level of DNA methylation in 12 genes was assessed in DNA extracted from the sputum of 184 smokers with asthma and compared with 511 controls that smoked but did not have asthma. The genes were selected for analysis due to their role in oxidative stress and DNA

repair pathways. A significant increase in DNA methylation levels was found in the protocadherin-20 (*PCDH20*) gene in asthmatic adults compared with control subjects. This gene is involved in cell adhesion and signal transduction (Sood et al., 2012). Recently, Joubert *et al*. performed a meta-analysis across 13 cohorts using the Illumina 450K and reported that maternal smoking during pregnancy was associated with differential DNA methylation in newborns at specific loci. Several of them were associated to diseases that can be caused by maternal smoking e.g. asthma (Joubert et al., 2016).

1.4.4.2. Effect of air pollution on DNA methylation in asthma patients

Exposure to traffic-related air pollution has long been involved with childhood asthma development through DNA methylation changes. Treg cells play a pivotal role in suppressing immune responses, and affecting the mechanism of allergic sensitization and inhibition of IgE production once exposed to allergen (Taylor et al., 2005). It has been shown that the numbers of Treg decreased in the bronchoalveolar lavage fluid of asthmatic patients. A study among asthmatic and non-asthmatic school children living in high and low pollution areas demonstrated that children with greater exposure to ambient air pollution have increased methylation levels of CpG islands in the *FOXP3* gene, which affects the function of Treg cells and increases the severity of asthma (Nadeau et al., 2010). This result was supported by a recent study in which Hew *et al.* found significantly increased *FOXP3* DNA methylation and decreased expression in Treg cells, and increased *IFNγ* protein expression in Teff in asthmatic children exposed to polycyclic aromatic hydrocarbons (PAH), a ubiquitous environmental pollutant released in the air during burning of organic matter such as coal, oil, petrol, and wood (Hew et al., 2015). Exposure to PAH during early embryonic development can cause changes in DNA methylation status. Perera *et al.* reported that exposure to airborne PAH during pregnancy resulted in increased methylation levels of CGIs in the acetyl-CoA synthetase long-chain family member 3 (*ACSL3*) gene in 81% of asthmatic children born to mothers with excessive PAH exposure. This gene is involved in fatty acid metabolism (Perera et al., 2009).

1.5. Sexual dimorphism in predisposition to disease

All species represent bias between males and females in anatomical, physiological, and behavioral susceptibility to diseases due to sex chromosome complements (females have two X

chromosomes, whereas males have one X and one Y) (reviewed in (Kulathinal, 2001, Rigby and Kulathinal, 2015)). In human genetics, sex is indicated by the presence or absence of the sex-determining region of the Y chromosome (SRY) region during the early stages of embryogenesis (Ober et al., 2008). The differences between two sexes then extend to cover different aspects, including hormonal changes, gene expression, and epigenetics, including DNA methylation. Such dimorphisms at both phenotypic and molecular levels have essential advantages to understand differences in disease pathogenesis, progression and treatment (reviewed in (Arnold et al., 2016)). Sexual dimorphism in selected aspects are briefly summarized below.

Gender differences are demonstrated in disparities in the prevalence, age of onset, severity and response to treatment between men and women. Generally, the prevalence of autoimmune disease is higher in females than males. Nevertheless, there are some exceptions (Ober et al., 2008). Women are more susceptible to multiple sclerosis (MS) than men, and the difference in that ratio recently increased to reach 3:1 (female: male) among Canadians. Such a dramatic change could be attributed to changes in environment and lifestyle (Ramagopalan et al., 2010). Systemic lupus erythematosus (SLE) is considered one of the very high incidence of autoimmune diseases that affected more women than men, the ratio is (9:1). However, male patients present significantly more disease severity compared to female patients (Crosslin and Wiginton, 2011, Borchers et al., 2010) (reviewed in (Ngo et al., 2014)). Asthma is an autoimmune disease that has had an exponential increase globally in its prevalence, morbidity, mortality, and economic burden. Several epidemiological studies discussed the presence of sexual dimorphism in asthma prevalence and severity and how age plays an important role. In childhood, males are more likely to develop asthma than females, whereas in adulthood, women have a higher risk of developing asthma than men (Postma, 2007).

Another example of sexual dimorphism in disease is the abdominal aortic aneurysm (AAA). It has been observed that males manifest AAA more frequently than females, with a male to female ratio of about 6:1. There are several reasons underlying the apparent sexual dimorphism in AAA, including lifestyle and sex hormones (reviewed in (Bloomer et al., 2012)). It has been suggested that increased circulating levels of testosterone are associated with AAA (Yeap et al., 2010).

Women and men respond differently to drug treatments, and understanding these differences is essential to designing safe and effective medications. Sexual dimorphism could be in any pharmacokinetic aspect, including drug absorption, distribution, metabolism, and excretion (Whitley and Lindsey, 2009). Women develop diseases such as major depression, anxiety, and thoughts of suicide more commonly than men, while men are more susceptible to Parkinson's disease (PD), attention-deficit hyperactivity disorder (ADHD) and autism spectrum disorders (ASD). Examples of drugs that illustrate sex differences include selective serotonin reuptake inhibitor (SSRI) antidepressants, for which females show a significantly greater response than males (Khan et al., 2005). It has been demonstrated that men are less sensitive than women to opioid receptor agonists, for example, morphine and kappa (OP2) receptor agonists. For that reason, usually men require 40% more doses of opioid analgesics than women to reach to the same degree of pain relief (Whitley and Lindsey, 2009).

1.5.1. Sex-specific differences in gene expression

Females and males often show dramatic differences in phenotype and behaviour, and these differences have been characterized in multiple species, including humans. Most of these differences are collectively attributed to sex bias in gene expression (reviewed in (Rinn and Snyder, 2005, Grath and Parsch, 2016)). Differences in gene expression between males and females are not limited to the mammalian genome and are found even in *Drosophila melanogaster.* Jin *et al*. (2001) used microarrays to assess the differences between two sexes in *D. melanogaster* at the transcriptional level and detected an interaction between sex, age and genotype. They noticed that gene expression was influenced more by sex than either genotype or age (Jin et al., 2001). Sex bias in gene expression has been found in other species, including *Xenaopus* (Malone et al., 2006), mice (Nalls et al., 2011) and *Caenorhabditis elegans* (Jiang et al., 2001). Examined tissue is considered a major determinant of sex-biased in gene expression, i.e. thousands of autosomal genes showed sex-biased in expression level between the sexes in several somatic tissues, levels of sex differences ranged from 14% in (brain) to 70% in (liver), in this case a gene may be showed biased in expression between males and females in some but not in other tissues (Yang et al., 2006) (Catalan et al., 2012). Moreover, sexual dimorphism in gene expression tends to increase in the gonads compared to other tissues, such as the brain (Wong et al., 2014). The influence of environmental factors on sexual dimorphism in gene expression has been shown in *D.*

melanogaster. Wyman *et al.* found that fruit flies reared under good nutritional conditions demonstrated sex-biased gene expression compared to those fed a poor diet (Wyman et al., 2010). A few examples illustrating sex-biased gene expression in various human tissues are briefly summarized here.

Several studies have focused on characterizing sex bias in gene expressions in different brain regions through development, adult life, and aging. Weickert and colleagues used a microarray approach to survey the expression of over 50,000 transcripts in the prefrontal cortex of humans and reported sex-specific differences in the expression of 130 genes, about 25 of which exist on the sex chromosomes (Weickert et al., 2009). Consistent with previous studies, Xu *et al.* performed genome-wide expression analysis in post-mortem prefrontal cortex human samples using gene expression BeadChips. They also noticed sexual dimorphism in gene expression in 35 genes (Xu et al., 2014). A more recent study identified gene expression differences between males and females throughout the developmental stages of the human brain. They indicated that there is upregulation in genes associated with schizophrenia, Alzheimer's disease and autism in male brains compared to female brains at different developmental stages. These results may explain the higher incidence of these diseases in men (Shi et al., 2016).

Another study used microarray analysis to assess sex-based differences in the transcriptome of human blood. They found that 582 autosomal genes showed sex-specific differences expression. About 58% of these genes were highly expressed in females and were found in genes associated with rheumatoid arthritis and other autoimmune diseases as well as genes regulated by estrogen, which emphasizes the importance of studying the sex bias in gene regulation (Jansen et al., 2014).

Sex-dependent differences in gene expression are well characterized in the human liver. In genome-wide studies using human liver samples to assess small expression differences, more than 1,200 genes were reported that show sexual differences in their expression, the majority of which are located on sex chromosomes, while others are on autosomes such as chromosome 9 (Zhang et al., 2011b). These genes are involved in diverse biological functions such as transcription, chromatin modification, lipid metabolism, and cardiovascular disease (Zhang et al., 2011b).

1.5.2. Sex-specific differences in DNA methylation levels

Of the many epigenetic mechanisms that have been studied thus far, DNA methylation has been shown to play an important role in gene expression and the regulation of cell differentiation. Recently, DNA methylation was included in a growing number of studies focused on genome-wide assessments in humans. DNA methylation differences have been noticed between sexes, and loci showing sex bias in DNA methylation were not exclusively found in sex chromosomes but also autosomes (Eckhardt et al., 2006). El-Maarri *et al.* found women had statistically lower methylation levels than men in the repetitive elements LINE-1 and Alu repeats as measured from whole blood samples in healthy subjects (El-Maarri et al., 2007). A few years later, El-Maarri and colleagues studied the effects of hormones like estrogen and progesterone on the DNA methylation at the LINE-1 repeats in four cell lines, and they found that methylation of LINE-1 was independent of the influence of these hormones (El-Maarri et al., 2011). In agreement with El-Maarri, Zhang *et al.* also found that leukocytes in females have a significantly lower level of global DNA methylation than in males (Zhang et al., 2011a). Quantitative profiling of DNA methylation was encountered in the white blood cells of random subjects to assess a small panel of four autosomal genes, which revealed a significant sex difference in DNA methylation in three out of four autosomal genes, with males showing higher methylation levels (Sarter et al., 2005).

Another study assessed the methylation of DNA found in saliva taken from 197 individuals, and they analyzed over 20,493 CpG sites using the Illumina Infinium assay. They found females sex bias in the DNA methylation of genes on the X chromosome as well as autosomes. These genes are involved in several biological mechanisms such as DNA transcription, RNA splicing, and cell-to-cell adhesion. Moreover, they noticed that DNA methylation was also affected by other phenotypes including age and smoking. They suggested that sex-specific methylation patterns are also site-specific (Liu et al., 2010a). Tapp and colleagues reported that in 185 healthy subjects, the influence of age-related CGI methylation on human rectal mucosa was not limited to sex, but also extended to folate availability and vitamin D. They further noticed that the levels of CGI methylation were higher in older men compared to women of the same age (Tapp et al., 2013). In an analysis of 280 CpG sites assessed from peripheral blood from 46 monozygotic and dizygotic twins and 96 healthy controls, Boks *et al.* found modest but significant sexual dimorphism in DNA methylation levels in the autosomal regions. This low difference in methylation levels between males and females could be due to the heterogeneity of the tissue type (Boks et al., 2009). Hall *et al.* found a sex bias in genome-wide DNA methylation in human

pancreatic islets, and they reported 470 autosomal regions and over 8,000 X chromosome sites that showed differences in DNA methylation levels. Additionally, they found 18 of these autosomal sites and 61 of the X chromosome loci showed sexual dimorphism in both DNA methylation and expression levels, revealing the potential role of DNA methylation in sex-specific metabolic differences (Hall et al., 2014).

McCarthy and colleagues conducted a meta-analysis of 76 other studies all using the 27 K BeadChip array to assess sex bias in DNA methylation in both autosomal and sex chromosomes across specimens, mainly blood and other tissues. They found 184 autosomal CpG sites that were differently methylated and were influenced by sex, the average difference in methylation level in percent was small, 3.7%. However, it was in concordance with previous studies, they also found that the autosomal sex bias in global DNA methylation was higher in males, while females had higher methylation levels in the X chromosome (McCarthy et al., 2014). A recent study identified 1,184 CpG sites showing differences in DNA methylation levels between sexes across autosomal regions by using a 450 K BeadChip array. Most of these CpG sites located in CGI shores and were enriched at imprinted genes, some of which were associated with altered gene expression (Singmann et al., 2015). Yousefi *et al.* performed a recent study in umbilical cord blood samples from males and females to analyze sex differences in DNA methylation using the 450 K BeadChip, they found over 3,000 CpG sites located in autosomes. From those, about 83% showed female sex bias DNA methylation, and most of the sex-biased autosomal CpG sites were found in regions associated with nervous system development and behavior. Furthermore, they searched for sex-associated DMRs and reported almost 76% of DMRs showed higher methylation in females (Yousefi et al., 2015).

1.5.3. Causes for sex-specific differences in DNA methylation in autosomal regions

Generally, sex bias has been attributed, in large part, to differences in hormonal levels between men and women. However, until recently, the genetic contribution to these sex-specific differences was less widely investigated. Genes on sex chromosomes and autosomes are now believed to contribute to multiple sexual changes in phenotypes (Ober et al., 2008, Wijchers and Festenstein, 2011). There are several approaches to reveal the origins of sex differences and to determine whether they are due to a sex complement mechanism or gonadal hormones (Arnold et al., 2012). One method involves manipulating the *SRY*, which is responsible for the initiation of

male sex determination in the non-gonadal cells of humans, and then assessing whether there is an influence on male-specific effects of this gene without *SRY* influences (Becker et al., 2005, Arnold et al., 2012). A second method is gonadectomy, which can be used to determine whether the sex bias was due to the influences of gonadal steroids at the time of testing. In gonadectomy, the gonads of males and females are extracted and the trait of interest is assessed. In controls, sham gonadectomy is an important step and involves removed the gonads and replacing them with another pair. If the trait changes after gonadectomy in one sex, then the conclusion is that gonads are essential for the investigated trait in that sex (Becker et al., 2005, Arnold et al., 2012). A third approach is the Four Core Genotypes (FCG) model that is used to investigate: (1) whether the sex differences in a trait are attributable to the influences of sex chromosomes after gonadal differentiation (XX vs. XY), or (2) whether the sex differences in a trait are attributable to the influences of gonadal secretions. FCG is based on the manipulation of the X and/or Y chromosome dosage. To create FCG mice, an (XY^-) female mouse that has the *Sry* gene deleted from the Y chromosome and has ovaries is mated with an (XY^-*Sry*) male mouse that carries the *Sry* transgene, which is inserted into an autosome, leading to progeny with one of four genotypes: XY^-*Sry* male, XX*Sry* male, XY^- female, and XX female (Arnold et al., 2012, Becker et al., 2005) (reviewed in (Arnold and Chen, 2009)).

It is well known that differences in phenotypes between females and males is largely driven by variations in gene expression levels, which are regulated by genetic and/or epigenetic factors such as DNA methylation. The exact nature of when and how sexual dimorphism in DNA methylation begins remains to be investigated. Selected examples about the influence of sex hormones and sex chromosomes in sex-biased DNA methylation and gene expression are summarized below.

In a recent study, genome-wide analysis was used to characterize sex-biased DNA methylation in a tissue-specific manner in mice. They found that DNA methylation is similar in male and female mouse livers during birth. They found decreases in methylation levels of tissue-specific enhancer sequences in adult male mice compared to females as well as in male embryos. Strikingly, when they assessed the DNA methylation in castrated male mice before puberty, they found hypermethylation of these regions. When the mice were provided with testosterone following castration, the demethylation levels were restored to normal, which suggests that

testosterone induces long-term, stable, sex-biased demethylation in male livers at the time of puberty (Reizel et al., 2015). El-Maarri *et al.* investigated whether the sex-specific variations of LINE-1 methylation were due to the effects of age or hormone variations. They showed decreases in LINE-1 DNA methylation levels in the female breast cancer T47-Kbluc cell lines after estrogen treatment (El-Maarri et al., 2011). It has been shown that estrogen receptor α (ERα) promoter methylation within the developing rat preoptic area (POA) is influenced by maternal-pup interactions such as licking and grooming in a sexually dimorphic manner. Males showed increases in the methylation levels of ERα promoters compared to females, which correlated with lower expression levels of ERα in males. In neonatal females, exposure to estradiol during brain development led to increased methylation of ERα (Kurian et al., 2010). The same finding was also confirmed within the developing rat amygdala (Edelmann and Auger, 2011). Ghahramani and colleagues conducted a genome-wide methylation analysis to examine sexual dimorphism and the influences of neonatal testosterone on the DNA methylation of the striatum and POA of mice. POA and striatum tissues were collected from postnatal and adult males and male controls as well as females controls and females treated with testosterone at birth. They found that exposure to testosterone at early stages of development leads to masculinized DNA methylation levels late in adulthood. Most of the genes influenced by the testosterone treatment showed increases in methylation levels (Ghahramani et al., 2014). It has been shown that prenatal exposure to the estrogenic endocrine disruptor substance bisphenol A (BPA) causes sex-specific changes in *Dnmt1* and *Dnmt3a* expression in juvenile cortex and hypothalamus. Furthermore, DNA methylation assessment of Erα also showed sex-specific and brain region-specific differences in DNA methylation of the estrogen receptor 1 (*Esr1*) in mice. Male cortex showed increased *Esr1* DNA methylation and consequently, reduced expression (Kundakovic et al., 2013).

There is growing evidence to suggest that a role for nonsteroidal influences (sex chromosome complement) in sex differences in gene expression and epigenetic mechanisms like DNA methylation. Carruth *et al.* used the Four Core Genotypes (FCG) tools to investigate whether sex chromosomes contribute directly to sex bias in mouse brain development. They found that dopaminergic neurons developed more within cultures containing one X chromosome (such as XY^{-} or XY^{-}*Sry*) than those derived from two X chromosomes (i.e. XX or XX*Sry* cells), suggesting that the development of dopaminergic neurons in mice can be independent of steroidal influences and instead related to sex chromosome effects (Carruth et al., 2002). Another study also used the

FCG method to test whether sex chromosomes influence sexual differentiation in the brains and behavior of mice. They showed that vasopressin fiber density in the lateral septum was more masculinized in XY^{-}*Sry* males than in XX*Sry* males. In addition, there was a noticeable difference in behavioral parameters, such as sniffing behavior, between XY and XY^{-}*Sry* males, demonstrating that both sex hormones and sex chromosomes are essential for sex differences in brain wiring and behavior (De Vries et al., 2002). Several phenotypes, such as the latency to exhibit aggression, parental behavior and pup retrieval, have been shown to be affected by both gonadal sex and sex chromosome complement (Gatewood et al., 2006). Zvetkova *et al*. noticed that in the absence of gonadal hormones, XX embryonic stem (ES) cell lines showed a reduction in DNA methylation globally and in the DMRs of imprinted genes compared to XY and XO lines *in vitro*. They showed that the reduction in DNA methylation was due to decreased levels of *Dnmt3a* and *Dnmt3b*. They suggested that there is a modifier encoded by the X chromosome whose product influences *de novo* methyltransferases (Zvetkova et al., 2005).

Wijchers *et al.* found that in a position effect variegation mouse model, where human *CD2* (*hCD2*) is integrated into a heterochromatic manner and shows variable silencing in T cells, the transgene is silenced in males more strongly than it is in females. In order to distinguish between genetic and hormonal influences on heterochromatic gene silencing, the *hCD2* transgene was crossed with the FCG mice (Wijchers et al., 2010). They found that transgene silencing occurred at a higher level in XY^{-} females and XY^{-}*Sry* males compared with XX females and XX*Sry* males, suggesting that the silencing could be due to sex chromosome complement through either a repressive influence of the Y chromosome or the overwhelming influence of two X chromosomes, rather than the hormones or sex phenotype. To test the repressive influence of Y chromosomes on silencing, the authors used another two mouse lines: XX^{Y*} males (where a Y* is attached to an X chromosome) and XO females. They showed that the expression of the *hCD2* transgene was at a higher level in XX^{Y*} males compared to XO females, indicating that the extent of gene silencing relies on X chromosome complementation independent of the presence or absence of the Y chromosome. They also found that sex chromosome complement also influences expression of autosomal genes in FCG mice. Furthermore, autosomal gene expression is affected by the presence of the *Sry* gene, they noticed that several genes sensitive to the sex chromosome complement phenomenon were increased in expression in XY^{-} females compared to XY^{-}*Sry* males (Wijchers et al., 2010, Wijchers and Festenstein, 2011).

Mutations in Lysine-specific demethylase 5C (*KDM5C*) are one of the leading causes of X-linked intellectual disability (XLID). *KDM5D* is a Y-linked homologue for *KDM5C* in human and mice. It has been indicated that the expression of *Kdm5c* was higher in the brains of XX female mice than XY male mice (Xu et al., 2002). Xu *et al.* used the FCG mouse model to investigate whether this difference was because of the sex chromosomes or sex hormones. They found that the female bias in *Kdm5c* expression in the brain depends on the sex chromosome dosage rather than the gonadal sex of the mice. They also noticed that the expression of *Kdm5d* was lower than *Kdm5c* in undifferentiated and differentiated mouse neurons, suggesting that *Kdm5d* cannot compensate for the difference in *Kdm5c* levels between the two sexes (Xu et al., 2008a).

Grafodatskaya and colleagues assessed DNA methylation of blood samples from patients with intellectual disability and mutations in the X-linked *KDM5C* gene by using a genome-wide DNA methylation array approach. They found a significant loss of DNA methylation at specific genomic regions at CGIs, suggesting that there are epigenetic modifications that are influenced by gene mutations involved in the regulation of histone modifications. Furthermore, they found in blood and brain samples that females have significantly higher levels of DNA methylation than males at f-box and leucine-rich repeat protein 5 (*FBXL5*) and calcyclin-binding protein (*CACYBP*), whereas sex comb on midleg, drosophila, homolog of 1 (*SCMH1*) showed this variation only in the blood. They also found that this sex bias was caused by the dosage of sex chromosome complement and not due to the effects of sex hormones (Grafodatskaya et al., 2013).

Several studies discussed the essential role of non-gonadal mechanisms (sex chromosome dosage) to explain the sex bias in autosomal gene expression between males and females in somatic cells. Indeed, it has been indicated that *SRY* may have functions other than testes determination. *Sry* has been found to be expressed in the substantia nigra of the adult male rat. Knocking down *Sry* in male rats caused downregulation of tyrosine hydroxylase expression, the rate-limiting enzyme in the dopamine synthesis mechanism, leading to motor dysfunction (Dewing et al., 2006). *Sry* also has a modulatory role on autosomal gene expression in a sex chromosome in a complement-specific manner in mice (Wijchers et al., 2010).

The inequality in the genomic dose of X genes is solved by the X chromosome inactivation mechanism in female embryos, which equalizes the expression of X-linked genes between the male and female. Interestingly, about 15% of the X-linked genes that exist on inactive X

chromosomes have been shown to variably escape XCI and are expressed on both active and inactive X chromosomes (Cotton et al., 2013). It has been shown that genes that escape from X inactivation have reduced expression levels compared to their counterparts on the active X chromosome (Cotton et al., 2013).

KDM5C is a X-linked gene that escapes X-inactivation and belongs to the evolutionarily conserved KDM5 family. This family contains four protein members, KDM5A/B/C and D. *KDM5C* encodes a transcriptional regulator with di- and tri histone H3 lysine 4 (H3K4) demethylase activity (Grafodatskaya et al., 2013). It is expressed largely in many cell types including white blood cells. The *KDM5C* transcript was observed extensively at skeletal muscle and brain. Mutations in the *KDM5C* are considered to be a cause for X-linked intellectual disability (XLID) and have been implicated in autism spectrum disorder (ASD) and renal carcinoma (Jensen et al., 2010) (Dalgliesh et al., 2010, Adegbola et al., 2008). Outchkourov *et al.* noticed in *Kdm5c*-knockout embryonic stem cells, *Kdm5c* seemed to have dual function depending on the place of its interaction on chromatin and through gene-specific transcription factors such as ELK1 and c-MYC. In promoter regions, *Kdm5c* played repressive role at promoters by demethylase H3K4me3 and H3K4me2 which negatively regulates transcription. However, in enhancer regions, *Kdm5c* played an activator role at enhancers by removing H3K4me3/2 and enriching enhancer histone mark H3K4me1 (Outchkourov et al., 2013). It has been indicated that *Kdm5c* enriched in promoter regions that harbor CGI and high H3K4me3 in mice neurons. Knocking out *Kdm5c* caused both decrease and increase in gene expression. Although, the vast majority of genes were upregulated which confirmed *Kdm5c* role as transcriptional repressor (Iwase et al., 2016).

In my project, we focused on two chromosomal regions, 5q31 and 17q21, both of which are considered to be among the best replicated asthma-associated regions from genome-wide association studies (GWAS) and share certain characteristics. These regions were among the highest genome-wide significance allele-specific expression differences: in the 17q region, the asthma-associated allele shows increased expression of *ORMDL3* and *GSDMB* genes, and in the 5q31 region, the asthma-associated allele shows increased expression of *SLC22A5* genes. This suggests that cis-regulatory mechanisms may influence disease development in these regions. Both regions show sex specificity of genetic association: the association with asthma is stronger in males than females, and both are associated with several autoimmune disorders (Al Tuwaijri et al., 2016).

1.6. Hypothesis and Objectives

1.6.1. Goal

The primary goal of my project is to test the role of DNA methylation in a common disease by analyzing two asthma-associated regions.

1.6.2. Scientific questions

- Are the sex-specific genetic associations and/or the allele-specific expression differences in the 5q31 region caused by variation in DNA methylation in the same region?
- Does the sex bias in the genetic association of asthma result from sex-specific differences in DNA methylation patterns at regulatory regions in 17q21?
- Does genotype influence DNA methylation patterns in the 5q31 and 17q21 regions?
- Do sex-specific DNA methylation patterns at the *ZPBP2* promoter found in PBMCs depend on the dosage of sex chromosomes?

1.6.3. Hypothesis

We hypothesize that variation in DNA methylation level at regulatory elements within asthma-associated genomic regions 5q31 and 17q21 may act as a modifier of the effect of genotype on phenotype.

1.6.4. Objectives:

1. Determine the DNA methylation signature of the *cis*-regulatory elements of the 17q21 and 5q31 asthma-associated regions.

2. Determine the cause(s) of sex differences in DNA methylation levels in these regions.

TABLES

Table 1.1 Examples from the best-replicated childhood asthma-associated regions based on GWAS results.

Genomic region	*Chr*	*Reference*
CRB1, DENND1B* and *CLORF53	1	(Sleiman et al., 2010, Melen et al., 2013)
ANTXR1*, *PROC, HNMT* and *IL18R1	2	(Chan et al., 2015, Moffatt et al., 2010, Raje et al., 2015, Wan et al., 2012)
SLC22A5	5	(Moffatt et al., 2010, Tang et al., 2016)
HLA-DQ	6	(Lasky-Su et al., 2012, Moffatt et al., 2010)
IL33	9	(Savenije et al., 2014)
GSDMB, GSDMA and ORMDL3	17	(Moffatt et al., 2007, Halapi and Bjornsdottir, 2009, Zhao et al., 2015)

FIGURES

Figure 1.1 Chromosomal region 5q31 in humans. **A.** Position of 5q31 GWAS asthma associated region in humans. All features are shown in the context of the UCSC Genome Browser, assembly GRCh38/hg38 (http://genome.ucsc.edu).Chromosomal region 5q31 in humans. **B.** Two common cis-reguwlatory haplotypes in LCLs. HapC carries asthma associated risk alleles and associated to increase expression of *SLC22A5, SLC22A4* and *IRF1* and lower expression of *P4HA2* whereas, HapD (sum of all non-HapC haplotypes) and has opposite pattern of gene expression.

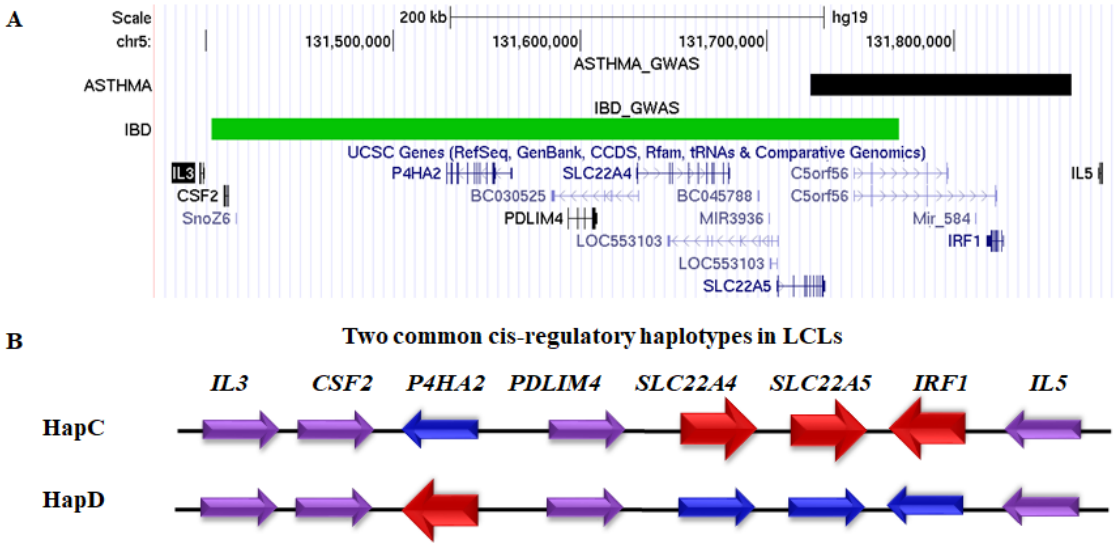
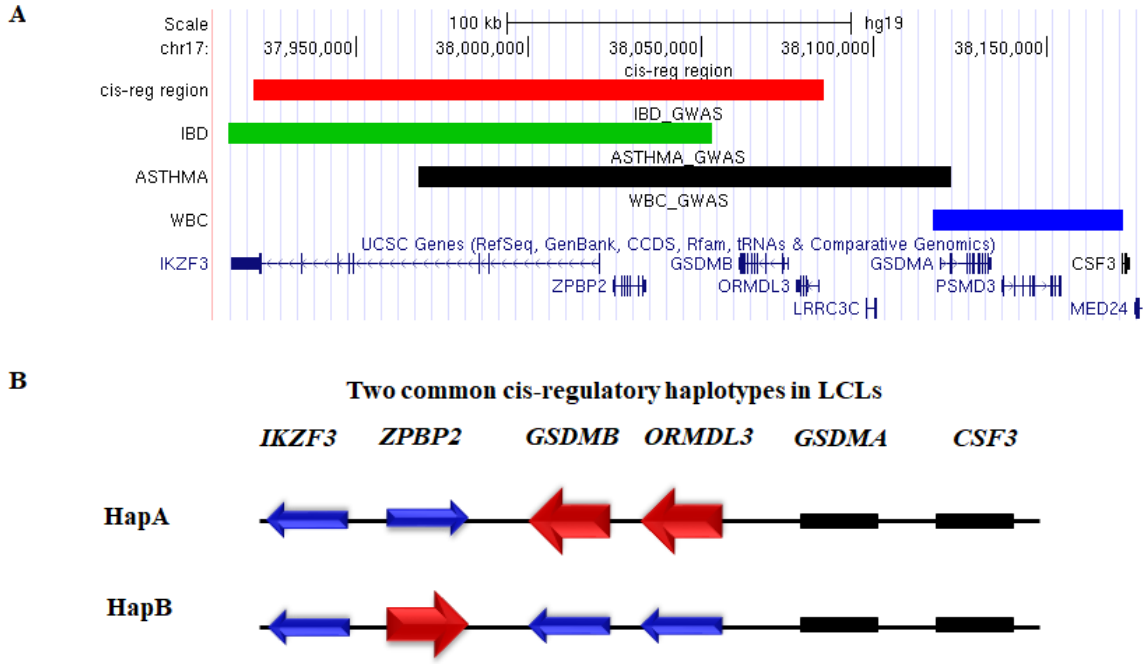


Figure 1.2 Chromosomal region 17q21 in humans. **A.** Relative positions of the *cis*-regulatory haplotype (red), the asthma-associated GWAS region (black), GWAS region associated with inflammatory bowel disease (green), GWAS region associated with neutrophil counts (purple). **B.** Two common cis-regulatory haplotypes in LCLs. HapA carries asthma associated risk alleles and associated to increase expression of *ORMDL3* and *GSDMB* and lower expression of *ZPBP2* and lower expression of *P4HA2* whereas, HapB (sum of all non-HapA haplotypes) and has opposite pattern of gene expression. *GSDMA* and *CSF3* is not expressed in LCLs.



CHAPTER 2: MATERIALS AND METHODS

2.1. Cell lines

Lymphoblastoid cell lines: DNA samples from the CEPH/UTAH HapMapPT01 panel of lymphoblastoid cell lines (LCLs) were purchased from the Coriell Cell Repositories (Camden, New Jersey, USA). Coriell website (https://catalog.coriell.org) provides the information about gender. LCL samples for methylation analysis were first selected for homozygosity in HapMap SNPs across the 500-kb region in 5q31 (Table 2.1). Among the 90 LCLs, four were homozygous for the asthma-associated haplotype (referred to as HapC) and two for non-HapC haplotype (referred to as HapD from this point on) alleles. To increase the number of samples, we added three cell lines that were heterozygous for one of the SNPs. (Table 2.1). For the 17q21 region, three LCL samples for methylation analysis were selected from HapA, HapB, and heterozygous HapMap SNPs across the 160-kb region in 17q21 (Table 2.2).

HEK293T/17: This is a hypotriploid human embryonic kidney epithelial cell line with a total of three copies of the X chromosome and four copies of chromosome 17. It is a derivative of the HEK293T; clone 17 was selected specifically for its high transfectability, wide availability, and good characterization (Table 2.3).

NuLi-1: The immortalized human lung epithelial cell line was derived from a 36-year-old male. NuLi-1 was generously provided by Dr. Simon Rousseau (McGill University) (Table 2.3).

Fibroblast cell lines: fibroblast DNA samples and fibroblast cell lines from individuals with different sex chromosome dosage were purchased from the Coriell Cell Repositories (Camden, New Jersey, USA). Coriell website (https://catalog.coriell.org) provides all information about sex, age, number of passages and presence or absence of *SRY*. When *SRY* genotype information was not available, fibroblast samples were genotyped for *SRY* by conventional polymerase chain reaction (PCR). Cell line description are listed in (Table 2.4).

2.2. Subjects

Recruitment and study design of the Saguenay-Lac-Saint-Jean (SLSJ) asthma familial collection was described in detail in (Laprise, 2014). Samples were provided by Dr. C. Laprise's Laboratory (University of Chicoutimi). Briefly, the SLSJ familial sample includes French-

Canadian probands with allergic asthma. The familial collection consists of initially recruited individuals and their families who joined the study. All participants of the study met the guidelines of the American Thoracic Society. Asthmatic participants were diagnosed with asthma if (1) the participant was diagnosed as having a history of asthma by a physician, or (2) the participant was diagnosed at the time of the recruitment with asthma-related symptoms and a positive PC20 provocative concentration, which causes a 20% drop in forced expiratory volume in one second (FEV1). Any participants that did not fulfill the criteria outlined above were considered unaffected. The work was done in accordance with the Canadian Tri-Council Policy Statement on Ethical Conduct for Research Involving Humans. The Centre de santé et de services sociaux de Chicoutimi, University Hospital local ethics committee, and the MUHC Research Ethics Board approved the study and all subjects gave their informed consent (Laprise, 2014, Naumova et al., 2013, Begin et al., 2007). Samples for *GSDMA*, *ZPBP2* and *SLC22A5*, pyrosequencing were selected according to their genotypes for the 17q21 region (asthma-associated haplotype HapA and non-HapA (HapB)) and the 5q31 region (HapC and D) and according to their familial link and their asthma, allergic and sex phenotypes. A description of the samples selected is shown in (Table 2.5).

2.3. Conventional polymerase chain reaction (PCR).

All DNA samples from fibroblast cell lines were genotyped for the *SRY* gene using conventional PCR. The 20µl PCR reaction contained 1µg fibroblast DNA, 2.5mM dNTP (BioLabs), 1.5mM MgCl2 (BioLabs), 10µM of each primer, and 0.1 units of *Taq* polymerase. The PCR conditions were as follows: At 95ºC for 3 min, followed by 35 cycles of 95º C for 45 s, 60º C for 30 s, 72º C for 1 min, and a final step of 72º C for 15 min. The PCR product was run in 2% agarose gels to test for DNA right size band (179bp). Primers are summarized in (Table 2.6).

2.4. Primer design for sodium bisulfite sequencing methylation assays

Nested PCR was performed for each locus in 5q31 and 17q21. Besides the general primer design recommendation, certain criteria should be considered when two sets of primers are designed for sodium bisulfite-treated DNA: (1) no CpG site or SNP should be located in the primer sequence; (2) the region should contain a common SNP, when possible (3) the two sets of primers should have very similar annealing temperatures (Tann); and (4) the 3’ prime end should contain

a converted base. Sodium bisulfite sequencing methylation assays primers were designed to amplify the promoter/exon 1 regions. In 5q31 region, *SLC22A5* and *PDLIM4* promoters were highly enriched with repeats; therefore, the assays were designed to amplify exon 1 CG-island shore or the upstream promoter region, respectively. *P4HA2* has two annotated alternative promoters; we assayed the promoter region of transcript variant 2. Primers are listed in (Table 2.6).

2.5. Sodium bisulfite sequencing methylation assay

One to two μg of DNA were treated with sodium bisulfite as described (Clark et al., 1994, Naumova et al., 2013, Berlivet et al., 2012). For most samples, at least two independent PCR reactions were done. PCR products were purified using the MinElute gel extraction kit (Qiagen, Hilden, Germany) and cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). The sequencing was done by the sequencing platform of the McGill University and Genome Quebec Innovation Centre. On average, 18 clones per sample were sequenced. Clone sequences were analyzed using the BiQ Analyzer software (Max-Planck-Institute for Informatics, Saarbrucken, Germany). The average percent of methylation was calculated for the region (number of methylated CGs vs. number of assayed CGs) and for each individual CG position. The proportion of unconverted cytosines was determined for each clone and clones with more than 2% unconverted cytosines were excluded from analysis. Characteristics of regions, primers and PCR conditions are summarized in (Table 2.6).

2.6. Treatment of cell lines with 5-aza-2-deoxycytidine (5-aza-dC)

The HEK293T/17 was cultured in Dulbecco's modified Eagle's medium (DMEM, 319-006-CL; Wisent Bioproducts, QC, Canada) at 37°C, 5%CO2, 100% humidity. Cultures were supplemented with heat-inactivated (10% v / v) fetal bovine serum (FBS, 080-450; Wisent Bioproducts, QC, Canada), penicillin-streptomycin solution (450-201-EL; Wisent Bioproducts, QC, Canada). HEK293T/17 cells were seeded in 6-well plates (1 x 10^5 cells/well) for 24h before being treated with 5-aza-2-deoxycytidine (5-aza-dC) (A3656-5mg; Sigma, Canada) which was dissolved in 50% acetic acid to obtain the concentration of 50mg/mL and stored in aliquots at −20°C, according to the manufacturer's instructions. The 5-aza-dC treatment was optimized to establish a working concentration, using a range from (20, 40, 50 and 100μM). The imprinted gene *H19* whose expression increases with promoter demethylation was used as positive control. Cell

survival and up-regulation of *H19* were used for selection of optimal conditions which was 50µM. The cells were treated with 50µM 5-aza-dC for seven days to allow the drug to be incorporated into DNA. HEK293T/17 cells were grown in triplicates for each condition, including a control group with no drug exposure i.e. HEK293T/17 (100µl 50% acetic acid without 5-aza-dC).

The NuLi-1 cell line was used until (passages 17-21) and cultured in Bronchial Epithelial Growth Medium (Lonza, Walkersville, MD) at 37°C, 5% CO_2, and 100% humidity. It was also supplemented with growth factors (SingleQuots (Lonza) and penicillin-streptomycin solution (450-201-EL; Wisent Bioproducts, QC, Canada). Cells were seeded in 60 x 15mm petri dishes (1.5 x 10^5 cells per plate) for 24h before treated with 5-aza-dC, which was dissolved in DMSO to obtain the concentration of 50 mg/ml. The 5-aza-dC treatment was optimized to establish a working concentration, using final concentrations in a range between 0.05 and 1 µM (0.05, 0.1, 0.5 and 1.0 µM). Imprinted gene *H19,* whose expression increases with promoter demethylation, was used as control for treatment efficiency. Cell survival and up-regulation of *H19* were used for selection of optimal conditions which was 0.5 µM. The cells were treated with 0.5 µM 5-aza-dC for 24h to allow the drug to be incorporated into DNA, the medium was changed and cells were grown for another seven days. NuLi-1 cells were grown in four replicates for each condition, including a control group with no drug exposure but 0.5% DMSO. RNA and DNA were extracted in the seventh day after the treatment. The images were captured using Leica DMI6000 B inverted microscope.

2.7. Transfection of HEK293T/17 cell line

HEK293T/17 was cultured in Dulbecco's modified Eagle's medium (DMEM, 319-006-CL; Wisent Bioproducts, QC, Canada). Cultures were supplemented with heat-inactivated (10% v⁄v) fetal bovine serum (FBS, 080-450; Wisent Bioproducts, QC, Canada), penicillin-streptomycin solution (450-201-EL; Wisent Bioproducts, QC, Canada). Cells were seeded in six-well plates (1 x 10^5 cells/well) for 24h before transfection. To reduce the levels of *KDM5C* RNA we used RNA small interfering (siRNA) approach KDM5C-specific siRNA (J-010097; Termo Scientific, Canada) RNAi sequences and locations are illustrated in (Table 2.7) (Figure 2.1). To determine the optimal siKDM5C concentration for the experiment, cells were transfected with 25nM, 50nM, 75nM, and 100nM of siKDM5C, whereas controls were treated with lipofectamine without siRNA. *KDM5C* expression was assessed. Cell survival and reduction of *KDM5C* RNA levels were used

to select the optimal conditions. After, we chose 50nM as an optimal concentration, cells were transfected with 50nM and incubated for different periods of time (48 h, 72 h, and 96 h). We found that the 72 h incubation showed the ideal cell survival and reduction of *KDM5C* RNA. SiRNA 50nM targeting *KDM5C* was transfected into HEK293T/17 cells (at 30-50% confluency) using lipofectamine-based chemical transfection (Lipofectamine 2000; Invitrogen, Carlsbad, Canada) according to the manufacturer's instructions. Lipofectamine without siRNA was added into HEK293T/17 as the negative control. RNA were extracted 72 h after the transfection.

To confirm the specificity of siKDM5C construct, *KDM5C* expression was assessed previously in HEK293T/17 transfected with siKDM5C, siCTCF (J-020165; Termo Scientific, Canada) and siP300 (J-003486; Termo Scientific, Canada) in separate. Lipofectamine without siRNA was added into HEK293T/17 as the negative control. The mRNA level of *KDM5C* was measured by quantitative RT-PCR and normalized to the GAPDH RNA levels in duplicate experiments (this part from the experiment was conducted by Dr. Nasser Fotouhi) (Table 2.7).

2.8. DNA extraction for sodium bisulfite sequencing methylation assays

DNA was extracted only when cells reached 80% to 90% confluence. The cell lines were incubated overnight at 55°C in 500 µl of buffer containing 50 mM Tris, pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% SDS and 5 µg of Proteinase K. The DNA was extracted using a standard phenol-chloroform procedure. DNA was quantified using the Nanodrop ND-2000 (Thermo scientific), and sodium bisulfite sequencing methylation assays were conducted.

2.9. RNA extraction and reverse transcription for expression analysis

RNA was extracted using Trizol (Life Technologies) when cells reached 80% to 90% confluence, and the concentrations were determined with the Nanodrop ND-2000 (Thermo Scientific). The reverse transcription reaction was performed with Invitrogen Oligo (dT) 12-18 primers and M-MLV reverse transcriptase according to the manufacturer's instructions. Briefly, 1 µg of RNA was used per reverse transcription reaction and treated with DNAseI enzyme. For each RNA sample, two independent RT reactions were prepared. The M-MLV enzyme was added to only one of the two tubes (RT+) while the other was used as a negative control (RT-). The reaction mix was incubated at 37°C for 50 minutes then at 70°C for 15 minutes to inactivate the enzyme.

To test for contamination, a conventional PCR using the ribosomal protein 18S housekeeping gene (*RPS18*) primers was conducted. PCR products were loaded on a 2% agarose gel.

2.10. Gene expression analysis

Primer3 software (v. 0.4.0) (http://bioinfo.ut.ee/primer3-0.4.0/) was used to design primers for quantitative real time PCR (qPCR). All cDNA primer sets were designed into two adjacent exons in order to detect for any possible DNA contamination. Primer sequences should not overlap with any known SNPs and should identify only the targeted genomic region, for that reason primers were blatted against the human genome assembly using the UCSC genome browser (GRCm37/hg19). Primers were validated using conventional PCR, Samples with no DNA contamination were used for expression analysis. List of primers for expression analysis is provided in (Table 2.6). The cDNA samples from cell lines were used to assess gene expression using qPCR. The qPCR was performed in a 10 µl reaction containing ABI CYBR-green using an ECO Real Time PCR System (Illumina). The qPCR program used for all tested genes was as follows: an initial step at 95°C (10min) and then 40 cycles of 95°C (20s), 60°C (45s), and 72°C (30s). Data were normalized by *RPS18* RNA levels and analyzed using the 2(-delta-delta C(T)) method (Livak and Schmittgen, 2001). The significance of changes in expression was determined using the Student's t-test.

2.11. Genotyping

Genotypes were established using PCR assays targeting SNPs in the *ZPBP2*, *ORMDL3*, *GSDMA*, *GSDMB* and *IKZF3* genomic regions followed by Sanger sequencing. Sequencing was done by the McGill University and Genome Quebec Innovation Centre sequencing service. PCR primers are listed in (Table 2.6).

2.12. Statistical analyses

Data for the effect of genotype on methylation levels were analyzed using Student's *t*-test two-tailed, and a p values $p \leq 0.05$ were considered significant. Error bars indicate standard deviation. Bonferroni corrections were applied to establish new significant threshold for p values according to the number of independent tests performed for each gene.

TABLES

Table 2.1 Genotypes of lymphoblastoid cell lines from the CEPH/UTAH HapMap panel that were selected based on their homozygosity across the 500-kb region in 5q31. Genomic coordinates are shown below the SNP id.

LCL/ Sex	*5q31 Haplotype*	*rs3091338* *131402738*	*rs1050152* *131676320*	*rs272867* *131681057*	*rs2631365* *131705949*	*rs2073643* *131723288*	*rs11739135* *131733397*	*rs2188962* *131770805*	*rs12521868* *131784393*	*rs2244012* *131901225*
NA12004 (F)	C	CC	CC	GG	CC	TT	GG	CC	GG	GG
NA12892 (F)	C	CC	CC	GG	CC	TT	GG	CC	GG	GG
NA12752 (M)	C	CC	CC	GG	CC	TT	GG	CC	GG	GG
NA07048 (M)	C	CC	CC	GG	CC	TT	GG	CC	GG	GG
NA07345 (F)	C	CC	CC	GG	CC	TT	GG	CC	GG	AG
NA12891 (M)	D	TT	TT	AA	TT	CC	CG	TT	TT	AA
NA10854 (F)	D	TT	TT	AA	TT	CC	CC	TT	TT	AA
NA10859 (F)	D	TT	TT	AA	TT	CC	CC	TT	TT	AA
NA12864 (M)	D	CT	TT	AA	TT	CC	CC	TT	TT	AA

Table 2.2 Genotypes of lymphoblastoid cell lines from the CEPH/UTAH HapMap panel across the 160-kb region in 17q21. Genomic coordinates are shown below the SNP id.

LCL/ Sex	*17q21 Haplotype*	*rs7216389 38069949*	*rs2872507 38040763*	*rs11078927 38064405*	*rs9303280 38074031*	*rs2290400 38066240*	*rs8069176 38057197*	*rs8067378 38051348*	*rs9303277 37976469*
NA11995 (F)	A	TT	GG	CC	CC	TT	GG	AA	CC
NA12761 (F)	A	TT	GG	CC	CC	TT	GG	AA	CC
NA12004 (F)	A	TT	GG	CC	CC	TT	GG	AA	CC
NA12239 (F)	B	CC	AA	TT	TT	CC	AA	GG	TT
NA12892 (F)	B	CC	AA	TT	TT	CC	AA	GG	TT
NA12006 (F)	B	CC	AA	TT	TT	CC	AA	GG	TT
NA12144 (M)	AB	CT	AG	CT	CT	CT	AG	AG	CT
NA12872 (M)	AB	CT	AG	CT	CT	CT	AG	AG	CT
NA12874 (M)	AB	CT	AG	CT	CT	CT	AG	AG	CT

Table 2.3 Cell line characteristics.

Cell line	*Cell type*	*Source*	*Diagnosis*	*Sex*	*Age*	*Immortalization*	*Karyotype*	*# of chr.17*	*Genotype at 17q21*
HEK293T/17	epithelial	embryonic kidney	normal	F	fetus	SV40	64, XXX isoXq, multiple anomalies	4	HapA
NuLi-1	epithelial	lung	normal	M	36	HPV, hTERT-Lxsn	46, XY, 24% of cells are polyploid, trisomy 5 and 20	2	HapAB

Table 2.4 Fibroblast cell lines DNA samples from Coriell Cell Repository (Information from https://catalog.coriell.org) and *SRY* genotype.

Fibroblast sample description	*Coriell ID*	*Karyotype*	*Sex*	*Age*	*Passage*	*SRY genotype*
Normal male	**GM02936**	**46,XY**	**M**	**20 Day**	**6**	**+**
Normal male	**GM03348**	**46,XY**	**M**	**10**	**7**	**+**
Normal male	**NA07753**	**46,XY**	**M**	**17**	**6**	**+**
Normal female	**NA00037**	**46,XX**	**F**	**18**	**16**	**-**
Normal female	**GM01652**	**46,XX**	**F**	**11**	**11**	**-**
Normal female	**GM00038**	**46,XX**	**F**	**9**	**10**	**-**
Turner syndrome	**GM00857**	**45,X.arr Xp22.33q28(108464-154887040)x1**	**F**	**19**	**5**	**-**
Turner syndrome	**NA02668**	**46,X,del(Y)(pter>q11.2:)[77]/45,X[33]**	**F**	**1 Day**	**5**	**+**
Turner syndrome	**NA00735**	**45,X.arr Xp22.33p11.1(108464-52927933)x1, Xp11.22q28(53086710-154887040)x2~3, 8p23.1(7254762-7825360)x1,22q12.3q13.33(32495977-49406499)x2~3**	**Ambiguous**	**41**	**4**	**-**
Turner syndrome	**NA01176**	**45,X**	**F**	**17**	**N/A**	**-**

Turner syndrome	**NA00088**	**46,X,idic(X)(p11.2).arr Xp22.33p11.21(108464-56474519)x1,Xp11.21q28(56,486,208-154,887,040)x3,5p15.2p15.1(14450134-15493849)x3,12p11.1(33420095-34694301)x3**	**F**	**19**	**5**	**-**
XXXXY male syndrome	**NA00157**	**49,XXXXY,t(4;11)(q35;q23)**	**M**	**28**	**12**	**+**
46,XY sex reversal SRY	**GM00048**	**46,XY (? H-Y antigen, ZFY+)**	**F**	**1**	**2**	**+**
46,XY sex reversal SRY	**GM01628**	**46,XY (H-Y antigen positive)**	**F**	**1**	**7**	**+**
46,XY sex reversal SRY	**NA03368**	**46,XY**	**F**	**17**	**6**	**+**
XX male syndrome	**GM02670**	**46,XX.ish der(X)t(X;Y)(p22.3;p11.3)(SRY+,DXZ1+).arr Yp11.31p11.2(2710425-5680476)x1**	**M**	**19**	**7**	**+**
XX male syndrome	**NA02626**	**46,XX.ish der(X)t(X;Y)(p22.3;p11.3)(SRY+,DXZ1+).arr Yp11.31p11.2(2710425-5680476)x1**	**M**	**23**	**4**	**+**
XX male syndrome	**GM01889**	**46,XX**	**M**	**24**	**3**	**-**
RETT syndrome; RTT	**NA07982**	**46,XX: MECP2 705delG**	**F**	**25**	**3**	**-**
RETT syndrome; RTT	**NA11271**	**46,XX: MECP2 mutation: No other data**	**F**	**2**	**3**	**-**
RETT syndrome; RTT	**NA11273**	**46,XX: MECP2 316C>T**	**F**	**11**	**7**	**-**

Table 2.5 Phenotypic description of the individuals selected from the Saguenay-Lac-Saint-Jean asthma familial collection for pyrosequencing analyses (Al Tuwaijri et al., 2016).

	SLC22A5			***GSDMA***			***ZPBP2***		
	HapC (n=6)	**HapCD (n=60)**	**HapD (n=618**	**HapC (n=17)**	**HapCD (n=60)**	**HapD (n=13)**	**HapC (n=37)**	**HapCD (n=60)**	**HapD (n=60)**
M: F ratio	1:0:5	1:1	1:0:38	1:0:89	1:1	1:16	1:1.06	1:1	1:1
Age, mean (range)	34 (6-51)	41 (10-70)^{D}	19 (5-46)^{CD}	32 (4-67)	41 (10-70)^{B}	24 (7-41)^{AB}	10 (5-16) ^{AB,B}	41 (10-70)^{A,B}	31 (5-70)^{A,AB}
Smoking status, n (%)*									
Never smoker	2 (33$^{)CD,D}$	26 (44)^{C,D}	16 (89)^{C,CD}	15 (88)^{AB,B}	26 (44)^{A,B}	9 (69)$^{A,\ AB}$	36 (97) ^{AB,B}	26 (44) ^{A,B}	40 (68) $^{A,\ AB}$
Ex- smoker	4 (67) ^{CD,D}	21 (36) ^{C,D}	0 (0) ^{C,CD}	1 (6) ^{AB,B}	21 (36) ^{A,B}	4 (31) $^{A,\ AB}$	0 (0) ^{AB,B}	21 (36) ^{A,B}	13 (22) $^{A,\ AB}$
smoker	0 (0) ^{CD,D}	12 (20) ^{C,D}	2 (11) ^{C,CD}	1 (6) ^{AB,B}	12 (20) ^{A,B}	0 (0) $^{A,\ AB}$	1 (3) ^{AB,B}	12 (20) ^{A,B}	6 (10) $^{A,\ AB}$
Differential white cell count (%), mean (SD)†									
Basophils	0.8 (0.4)	0.8 (0.4)	0.6 (0.5)	0.9 (0.5)	0.8 (0.4)	0.4 (0.5)	0.6 (0.5)	0.8 (0.4)	0.8 (0.7)
Eosinophils	3.4 (1.3)	3.6 (2.6)	5.4 (5.5)	5.6 (5.6)	3.6 (2.6)	3.0 (1.8)	5.5 (4.5)^{B}	3.6 (2.6)	3.3 (2.3)^{A}
Monocytes	7.8 (1.9)	8.3 (2.0)	8.2 (1.7)	8.7 (1.7)	8.3 (2.0)	7.3 (2.5)	7.9 (2.3)	8.3 (2.0)	8.3 (1.7)
Neutrophils	56.6 (9.4)	54.3 (8.1)	49.8 (9.6)	54.4 (11.9)	54.3 (8.1)	51.9 (17.9)	48.9 (7.7)^{HET}	54.3 (8.1)^{A}	52.4 (8.9)
FEV1, % pred. (SD)‡	92 (13)	94 (18)	99 (11)	94 (13)	94 (18)	98 (14)	99 (26)	94 (18)	95 (20)
PC20 (SD)§	5.16 (1.62)	10.12 (5.46)	14.56 (2.73)	7.58 (6.57)	8.99 (5.56)	5.7 (5.63)	6.38 (3.7)	10.12 (5.46)	6.29 (4.42)
IgE mg/L (SD)¶	191 (13)	97 (5)	100 (10)	135 (8)	97 (5)	142 (4)	196 (13)	97 (5)	177 (7)
Asthma, n (%)*	4(67)	30 (50)	11 (61)	9 (53)	30 (50)	6 (46)	23 (62)	30 (50)	30 (50)
Allergy, n (%)††	5 (83)	30 (50)	11 (65)	9 (53)	30 (50)	11 (85)	23 (64)	30 (50)	38 (63)
Asthma with allergy, n (%)	4 (67)	30 (50)	8 (47)	6 (35)	30 (50)	5 (38)	16 (44)	30 (50)	21 (35)

Significant differences between subsamples specific to each gene haplotype after $\chi 2$ or Kruskal–Wallis analyses are shown with capital letters in superscript. These letters indicate between which haplotype subsamples the differences are significant.

*Smoking status available for 83 individuals selected for SLC22A5, 89 for GSDMA and 155 for ZPBP2 genes. Ex-smokers are defined as subjects who stopped smoking since 1 year or more.

†The white blood cell counts calculated as mean percentages and SD for 77 individuals selected for SLC22A5, 84 for GSDMA and 140 for ZPBP2 genes.

‡The mean forced expiratory volume in 1 s (FEV1) calculated as % of predicted value (% pred.) and SD for 75 individuals selected for SLC22A5, 85 for GSDMA and 137 for ZPBP2 genes.

§The geometric mean calculated for the provocative concentration of methacholine that induces a 20% fall in FEV1 (PC20) and SD for 72 individuals selected for SLC22A5, 81 for GSDMA and 128 for ZPBP2 genes.

¶The geometric mean and SD for the serum immunoglobulin E level for 78 individuals selected for SLC22A5, 87 for GSDMA and 143 for ZPBP2.

**Present or past documented clinical history of asthma. The asthma phenotype is available for all individuals.

††Defined as at least one positive response on skin prick testing (wheal diameter ≥3 mm at 10 min). The allergy phenotype is available for all individuals.

Table 2.6 Primers sequences used in all assays.

Assay	*Genomic region*	*Chr*	*SNP*	*Position of SNP (hg19)*	*Genome coordinates (hg19)*	*Round of PCR*	*Annealing Temp. (°C)*	*Forward primer 5'-3'*	*Reverse primer 5'-3'*
Genotyping	*SRY*	Y	-	-	chrY: 2787078-2787257	-	60	TCTTGAGTGTGTGGCTTTCG	TACAGGCCATGCACAGAGAG
Bisulfite sequencing	*IL3*	5	rs40401	131396332	chr5:131396211-131396629	1st	50	AATTTTCTATAAAAATTCCATATCAA	AGGTTTTTTGGTTTTAGGTAGT
Bisulfite sequencing	*IL3*	5	rs40401	131396478	chr5:131396291-131396592	2nd	60	AAATTATAAACACCTTACTACTAC	GTTGTAGGTTAGTTTTATTTAAGT
Bisulfite sequencing	*CSF2*	5	None	-	chr5:131409374-131409854	1st	50	AAACCAAAAAATTCCACAATTCAA	GGGGTTTTTGGAAAGTTATGTT
Bisulfite sequencing	*CSF2*	5	None	-	chr5:131409461-131409829	2nd	60	AACTCTTTTACCAATAAACCCAA	GAGAAGTATTTTATTTGGAGGTT
Bisulfite sequencing	*P4HA2*	5	rs72793280	131562900	chr5:131562651-131563087	1st	50	CCTTTTACCAACCTTCCTCTA	TTGAGGGGTTTTTTTATATTGGT
Bisulfite sequencing	*P4HA2*	5	rs72793280	131562900	chr5:131562728-131563038	2nd	60	TAGGGTTTTTTAGGTAATGGG	TTGAGGGGTTTTTTTATATTGGT
Bisulfite sequencing	*PDLIM4*	5	None	-	chr5:131592782-131593188	1st	50	TTTTTTTTAGGAAGTTTTATTGGT	ACCCCCACTCAACTCTCCAA
Bisulfite sequencing	*PDLIM4*	5	None	-	chr5:131592816-131593188	2nd	60	TAGTTTGAGGATTTTTTTGTGTT	ACCCCCACTCAACTCTCCAA
Bisulfite sequencing	*SLC22A4*	5	None	-	chr5:131630087-131630778	1st	50	TAGGGAGTTTTAAGGGGGGT	AAATATACAATAACATCAAACTCAA
Bisulfite sequencing	*SLC22A4*	5	None	-	chr5:131630179-131630650	2nd	60	AGGTAGTTTTTTTGTTTTAGTTGT	CCAATTTCTAACAACCTACCTA

Bisulfite sequencing	*SLC22A5*	5	rs2631365	131705949	chr5:131705744 -131706256	1st	50	CAACTAAAACAAAAA AACTATCTA	ACTATCTAAATAACTAA AAATTCAA
Bisulfite sequencing	*SLC22A5*	5	rs2631365	131705949	chr5:131705744 -131706217	2nd	58	AGTATATTGTGGTTG GAGGTT	AAGTTATTTGGTTGGAG GGTT
Bisulfite sequencing	*IRF1*	5	None	-	chr5:131826323 -131826827	1st	50	AGTGGAAGAGGGAA GAAGGT	AAAAAATAACCCCAAA AACCAA
Bisulfite sequencing	*IRF1*	5	None	-	chr5:131826332 -131826827	2nd	60	GGGAAGAAGGTAGA GGTTGT	AAATAAAAAAACAAAC TAAAACCAA
Bisulfite sequencing	*IL5*	5	None	-	chr5:131878921 -131879480	1st	50	ACTCAAAATCTTATT ATTTATAACA	GTTAATTTTTGATTTTA AGAAATGT
Bisulfite sequencing	*IL5*	5	None	-	chr5:131878921 -131879466	2nd	60	AAATAATTACCTATA CACTTTACAA	TTTAAGAAATGTAAATG TGGGGT
Bisulfite sequencing	*IKZF3*	17	rs1453559	38020419	chr17:38020302 -38020853	1st	50	ACTCTTTCTACTACTT ACACAAA	TTATTATTGAATTTAGA TAAAAAGTTT
Bisulfite sequencing	*IKZF3*	17	rs1453559	38020419	chr17:38020312 -38020790	2nd	60	CTTACACAAATTAAA TTTCTCAAAA	TTTAGTTTGTTAAGAAA GTAAGGT
Bisulfite sequencing	*ZPBP2*	17	rs4795397	38023745	chr17:38023652 -38023987	1st	50	TGTATTTTGTTTGAAT TTTGGGTTT	TTTACCTAATTACATTC TTTACCAACAAA
Bisulfite sequencing	*ZPBP2*	17	rs4795397	38023745	chr17:38023682 -38023857	2nd	58	GGGTATGATTTTTTTT GGAGTTTTT	TTTACCTAATTACATTC TTTACCAACAAA
Bisulfite sequencing	*ZPBP2*	17	rs11557466	38024626	chr17:38024114 -38024777	1st	50	TCCTCAAATCCCCTTC CTAAA	TTTTTAGTTAGAAGAGG AGTAGGGTTTAG
Bisulfite sequencing	*ZPBP2*	17	rs11557466	38024626	chr17:38024255 -38024701	2nd	60	AACTTCTTTTCCTTTA TTCTCTTCCTAC	GGTAGAGGGAGAGGTT TGGG
Bisulfite sequencing	*GSDMB*	17	rs9303280	38074046	chr17:38073646 -38074181	1st	50	TTTATATTTGGTAGTT TGAGGTGTTAG	TAACCCTCTTTACTAAA TCATAACTAC

Bisulfite sequencing	*GSDMB*	17	rs9303280	38074031	chr17:38073659-38074099	2nd	60	GGTAGTTTGAGGTGTTAGGTTTTTG	ACCTTTAACTCTTCCTTAACCTCATATC
Bisulfite sequencing	*ORMDL3*	17	-	-	chr17:38083703-38084111	1st	50	AACCCTAAAACCTCTTAATTC	TATATTTGAAGGGTATAGAAGT
Bisulfite sequencing	*ORMDL3*	17	-	-	chr17:38083811-38084089	2nd	60	CTACTACTCCAACAACTATAA	TATATTTGAAGGGTATAGAAGT
Bisulfite sequencing	*GSDMA*	17	rs3902025	38119254	chr17:38083790-38084111	1st	50	TTGGTATTTGTTTGGTAGTTTTT	AACAACCTCCTCTTCACCAA
Bisulfite sequencing	*GSDMA*	17	rs3902025	38119254	chr17:38119129-38119479	2nd	58	TAGTTTTTAGGAGGTTAGGGT	CACCAAAAAAATATCCACAAAC
Bisulfite sequencing	*CSF3*	17	rs2227322	38171668	chr17:38171558-38171985	1st	50	GTATTGTTTAGAGTGTATTGTGT	TACATTATCTTAAACACCAAATTTA
Bisulfite sequencing	*CSF3*	17	rs2227322	38171668	chr17:38171571-38171965	2nd	60	GTTATAGTAGTAGTTGTAGGGT	ACACCAAATTTACATAAATCCTAA
Expression (RT-qPCR)	*RPS18*	6	-	-	chr6:4470240-4470488	-	60	TGTGGTGTTGAGGAAAGCAG	GGACCTGGCTGTATTTTCCA
Expression (RT-qPCR)	*H19*	11	-	-	chr11:2017207-2017375	-	60	TTACTTCCTCCACGGAGTCG	CTTGAGCTGGGTAGCACCAT
Expression (RT-qPCR)	*SLC22A5*	5	-	-	chr5:131724626-131726488	-	60	GTTCCAAGAAGCAGCAGTCC	ATGCTGGGACTTCAACCATC
Expression (RT-qPCR)	*SLC22A4*	5	-	-	chr5:131667448-131670506	-	60	GGAGCTAAATCCCCTGAAGC	TCAATCAAGGCAGAGAGGAAA
Expression (RT-qPCR)	*KDM5C*	X	-	-	chrX:53222723-53223020	-	60	TGGAGAATGGAGACAGTGTGA	CCATCATGAGCTCCTCCAAG

Expression (RT-qPCR)	*DNMT1*	19	-	-	chr19:10244229-10244381	-	60	CCGAGAGAGTGCCTCAGCTA	GCCACAAACACCATGTACCA
Expression (RT-qPCR)	*DNMT3A*	2	-	-	chr2:25456998-25457160	-	60	TGCGTGTGTGTAAGGGACAT	CGCCTCTGTGGTTTTTGTTT
Expression (RT-qPCR)	*FBXL5*	4	-	-	chr4:15606070-15606279	-	60	TGCGTTTTGCTTTATTGCAC	AACAACAATCACTGGCCACA
Expression (RT-qPCR)	*CACYBP*	1	-	-	chr1:174977808-174979200	-	60	CTGACCCAGGTTGAAAAGGA	TCCTTTGGCTTGCTTCTCTC
Expression (RT-qPCR)	*SCMH1*	1	-	-	chr1:41493923-41494337	-	60	ATGGACAGTCGAGGATGTG	CAGCTTCAGGCCCATGTACT
Expression (RT-qPCR)	*IKZF3*	17	-	-	chr17:37944558-37947763	-	60	ACTGCACACAGGGGAAAAAC	TTGTGCTCCTCAAGGGAACT
Expression (RT-qPCR)	*ZPBP2*	17	-	-	chr17:38031612-38032991	-	60	GTAGTACGTCTGGATAGCTGTCG	CGCAGGTCTGACAAGTTACAT
Expression (RT-qPCR)	*GSDMB*	17	-	-	chr17:38061182-38061742	-	60	TGAAGAGCAGCAGTTTGTGG	CAGCTCATCCCAGTTCTGC
Expression (RT-qPCR)	*ORMDL3*	17	-	-	chr17:38078259-38078441	-	60	GCAGCCAAAGCACTTTAACC	AAAAGCCTGGGACTTGGATT
Expression (RT-qPCR)	*GSDMA*	17	-	-	chr17:38119784-38119927	-	60	TCTGATGTTGGGGACGTACA	CCTCCAGGGTCACTTCATGT
Expression (RT-qPCR)	*CSF3*	17	-	-	chr17:38173761-38173961	-	60	GACATGGTTTGACTCCCGAACA	TTCACACACAGGCCTGACA
Allelic expression	*IKZF3*	17	rs907092	37922259	chr17:37944951-3794532	-	60	ATTCATTGGTGAGAAGCGCCA	GTGAATCGTGAACATCACATAG

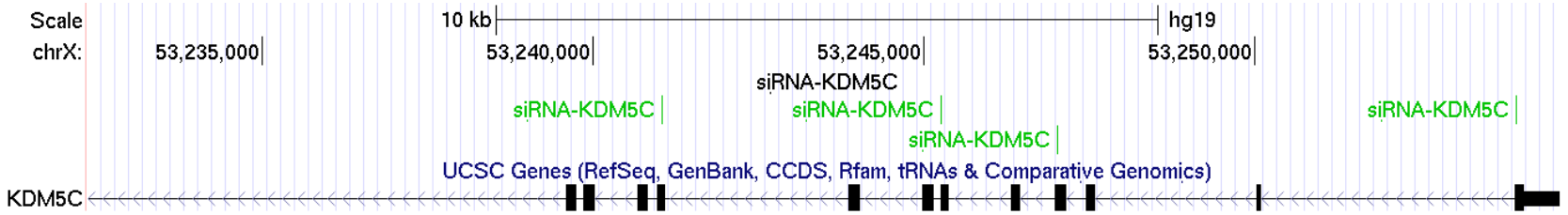
Allelic expression	*ZPBP2*	17	rs11557467	38028634	chr17:38027767-38028718	-	60	TTTTTGGAGCCTTTGTCTGG	TGTATGAGGCCATGTTCTGG
Allelic expression	*GSDMB*	17	rs2305480	38062196	chr17:38062142-38062457	-	60	CAAGGACCTGACAGAGGAGA	CTTCTACCAAGACCCCAGCA
Allelic expression	*ORMDL3*	17	rs12603332	38082807	chr17:38082702-38082915	-	60	GAGGGGGAAAGAGCTACCAG	ACTGGCCCCTACCCTAGATG
Allelic expression	*GSDMA*	17	rs7212944	38122686	chr17:38119207-38119312	-	60	AGTGGAGGGAGATGTGGATG	CCTTCAGGAATGGGTGGTCT

Table 2.7 siRNA sequences

Gene	*Smart poll siRNA*	*Sequences*
KDM5C	J-010097-09	GAGCAGGCUACCCGGGAAU
KDM5C	J-010097-10	UCGCAGAGAAAUCGGGCAU
KDM5C	J-010097-11	GACAAGACUCUGCGGAAGA
KDM5C	J-010097-12	CUACGAACGCAUUGUUUAU
P300	J-003486-11	GGACUACCCUAUCAAGUAA
P300	J-003486-12	GACAAGGGAUAAUGCCUAA
P300	J-003486-13	GUUCAAUAAUGCCUGGUUA
P300	J-003486-14	CGACAGGGAUGCAGCAACA
CTCF	J-020165-07	GAUGAAGACUGAAGUAAUG
CTCF	J-020165-08	GGAGAAACGAAGAAGAGUA
CTCF	J-020165-09	GAAGAUGCCUGCCACUUAC
CTCF	J-020165-10	GAACAGCCCAUAAACAUAG

FIGURES

Figure 2.1 *KDM5C* gene position. Location of siRNA primers in (green). All features are shown in the context of the UCSC genome browser, assembly GRCh37/hg19 (http://genome.ucsc.edu).



CHAPTER 3: RESULTS

3.1. Local genotype influences DNA methylation at asthma-associated region in *SLC22A5* at 5q31 region

3.1.1. Genotype influences DNA methylation levels at the *SLC22A5* gene promoter in 5q31 region of lymphoblastoid cell lines

To indicate whether allele-specific expression differences in the 5q31 region is associated with genotype-dependent methylation, as well as to detect genes with genotype-dependent differentially methylated regions (gDMRs), a pilot study was conducted to analyze DNA methylation of gene promoters in the 5q31 region of lymphoblastoid cell lines that were the basis for selecting target regions for further studies, i.e. pyrosequencing methylation assays that were later performed in a larger sample of patients by Dr. Laprise team. The HapMap CEU LCLs that were homozygous for either the HapC or HapD haplotype alleles were selected (Table 2.1). DNA methylation patterns of promoters and first exons of four genes, *SLC22A5*, *SLC22A4*, *P4HA2* and *IRF1*, with allelic expression bias in LCLs were analyzed. The other four genes *IL3*, *CSF2*, *PDLIM4* and *IL5* that did not indicate allele-specific expression differences in LCLs were used as controls. The effects of the genotype and sex of the cell donor (averaged across all interrogated CGs as well as individual CGs) on methylation levels were tested. Methylation analysis of the regulatory regions of 5q31 in DNA from LCLs is illustrated in (Table 3.1). If the AE differences found in the 5q31 region are due to DNA methylation, we expected to detect methylation differences between HapC and D homozygous LCLs. *SLC22A4* and *IRF1* promoters were hypomethylated (average methylation: 1% and 0.3%, respectively), and methylation status was independent of the genotype or sex of the donor respectively (p = 2.16E-01, p = 8.26E-01 for *SLC22A4*, p = 3.12E-01, p = 6.77E-01 for *IRF1,* Student's t-test). *IL3, CSF2, P4HA2*, *PDLIM4* and *IL5* promoter regions showed inter-individual variation in methylation levels. No statistically significant effect of genotype or sex on methylation level was detected for *IL3, CSF2, PDLIM4* and *IL5* (Table 3.1) (Figures 3.1-3.4) (Al Tuwaijri et al., 2016).

The *P4HA2* promoter showed an effect of haplotype on average methylation (Table 3.1) mostly driven by CG3 (hg19 chr5: 131562807), with an average methylation of 63% on HapC and 97% on HapD alleles (p = 1.10E-03, Student's t-test) (Figure 3.4), but not the other CGs. The CG3

effect remained significant after correction for multiple testing (*p* value significance threshold after correction was 4.5E-03) (Table 3.2) (Al Tuwaijri et al., 2016).

The *SLC22A5* region was hypomethylated for the most part, with no statistically significant effect of haplotype on average *SLC22A5* methylation levels across the 39 CGs (Figure 3.5A) (Table 3.1). However, when the methylation levels of individual CGs were considered separately, we found that HapC homozygotes had lower methylation levels at CGs 18, 26, 29, 30, 33 and 35 compared to HapD homozygotes (p = 1.3E-02, 2.3E-03, 2.0E-02, 3.3E-02, 1.0E-02 and 2.2E-02 respectively, Student's t-test) (Figure 3.5B). The significance of the haplotype effect was lost after correction for multiple testing (*p* value significance threshold after correction was 1.2E-03). We also found no effect of sex on average methylation across all 39 CGs (Table 3.1), but significantly higher methylation levels at CG5 (hg19 chr5: 131705809) in male samples (p = 8.0E-07, Student's t-test) (Table 3.3). Thus, of the eight promoter regions analyzed, data for *P4HA2* and *SLC22A5* are suggestive of a genotype effect on methylation; however, only at *SLC22A5* does the genotype influence methylation of several adjacent CGs, so the region may be considered a gDMR (Al Tuwaijri et al., 2016).

3.1.2. Genotype influences DNA methylation levels at the *SLC22A5* gene promoter in the 5q31 region of DNA samples from peripheral blood

It has been suggested that long-term cell culture, and using Epstein-Barr virus for immortalization affect DNA methylation patterns (Saferali et al., 2010, Grafodatskaya et al., 2010, Sugawara et al., 2011). We asked whether the genotype effect on *SLC22A5* methylation was also present in non-cultured cells. To answer this, seven CGs (CGs 31 to 37) (Figure 3.5B) were selected for DNA methylation analysis in 84 DNA samples extracted from peripheral blood from the SLSJ asthma familial collection using a pyrosequencing methylation assay in Dr. Laprise's laboratory. Among these DNA samples, six were homozygous for the rs272867-G allele (HapC), 60 were heterozygous, and 18 were homozygous for the rs272867-A allele (HapD).

The Hap D allele showed statistically significant increase of DNA methylation levels through the seven CG sites (CG31-CG37) (Table 3.4). We did not find significant differences in methylation levels between males and females (from p = 2.4E-02 to p = 2.4E-01). Then, we tested the correlation between methylation levels at individual CGs, we noticed correlations between five

out of seven CGs using Pearson's correlation tests (correlation coefficients between 0.5 and 0.9) (Al Tuwaijri et al., 2016). Then, the methylation levels of every individual CGs were analyzed separately. We found that even after each CGs were considered, the HapD homozygous samples remained associated with increase methylation level among every single CG of the seven CG sites compared to the HapC samples (p values from p = 1.2E-05 to p = 4.2E-10). Heterozygous samples, showed intermediate variation in DNA methylation levels at CGs 36 and 37, at CG 31 the methylation level was low, close to HapC homozygotes, while at CGs 32, 33, 34 and 35 the methylation levels were high close to HapD homozygotes (Table 3.4).

The influence of asthma on *SLC22A5* DNA methylation was studied in Dr. Laprise's laboratory, they did not find any significant effect, for more details (Appendix A.1) (Al Tuwaijri et al., 2016).

3.1.3. 5-aza-2'-deoxycytidine (5-aza-dC) treatment influences *SLC22A5* gene expression but does not change the methylation level of the promoter/exon 1 region

Next, we assessed whether the moderate changes in DNA methylation leads to a change in the expression levels of *SLC22A5 in vitro*. HEK293T/17 cells were treated with the DNA methyltransferase 1 (*DNMT1*) inhibitor, 5-aza-dC. Cells were harvested for RNA and DNA extraction. Imprinted gene *H19*, that showed upregulation with promoter demethylation, was used as a positive control to monitor the effectiveness of 5-aza-dC treatment. Cell survival and up-regulation of *H19* were used for selection of optimal conditions. Treatment with 50 and 100µM of 5-aza-dC led to increased *H19* expression, but the 100µM 5-aza-dC treatment also induced massive apoptosis. Therefore, we chose 50µM 5-aza-dC for the next experiments, as it showed both *H19* upregulation and reasonable cell viability (Figure 3.6A). We observed an 11-fold increase in the expression level of *H19,* which indicated that HEK293T/17 was successfully demethylated by 5-aza-dC (Figure 3.7A). There was a modest but statistically significant increase (1.5-fold) in *SLC22A5* expression after 5-aza-dC treatment (Figure 3.7A). To investigate if this effect was due to demethylation of the *SLC22A5* promoter region, DNA was extracted from treated cells and untreated controls, and the *SLC22A5* promoter-exon 1 region methylation level was assessed by the sodium bisulfite sequencing methylation assay. In contrast to LCLs, the *SLC22A5* promoter region including the CG-island shore, was hypomethylated in HEK293T/17 controls. No major

difference in DNA methylation was found between controls and 5-aza-dC treated cells (average methylation: 0.5% and 0.7%, respectively) (Figure 3.7B), suggesting that DNA methylation is not modulating the expression levels of *SLC22A5* in HEK293T/17 cell line.

A lung epithelium cell line, NuLi-1, was also treated with 5-aza-dC. As with HEK293T/17, cells were harvested for RNA extraction. To determine the optimal 5-aza-dC concentration for the experiment, cells were treated with 1μM, 0.5μM, 0.1μM, and 0.05μM of 5-aza-dC, while controls were treated exclusively with 0.5% DMSO. Imprinted gene *H19* expression was used as a positive control to identify the optimal concentration of 5-aza-dC. Treatment with 1μM and 0.5μM 5-aza-dC provided the highest upregulation of *H19*, but the 1μM 5-aza-dC led to massive cell death. Therefore, 0.5μM 5-aza-dC was chosen for our next experiments, as it showed both *H19* upregulation and reasonable cell viability (Figure 3.6B). We observed a five-fold increase in the expression level of *H19*, which indicated a successful demethylation of NuLi-1 (Figure 3.7A). As in HEK293T/17, there was also a modest but significant increase (1.6-fold) in *SLC22A5* expression after 5-aza-dC treatment (Figure 3.7A).

3.2. Sex, and genotype influence DNA methylation at the 17q21 locus associated with childhood asthma

3.2.1. Design and validation of sodium bisulfite sequencing methylation assays for seven regulatory regions in 17q21 in DNA samples from CEU LCLs

Assays for *ZPBP2, ORMDL3* and *GSDMB* were established previously by other members of the laboratory (Berlivet et al., 2012). These three genes showed allele-specific differences in expression levels in LCLs (Verlaan et al., 2009). The effect of genotype on methylation levels in the promoter/exon1 regions of the 17q21 genes was tested in LCLs to validate the assays. Validation of methylation profiles was conducted in the SNP rs4795397 region and the *IKZF3, GSDMA,* and *CSF3* genes. The *IKZF3* and the rs4795397 regions were unmethylated in all tested cell lines independent from their genotypes (Berlivet et al., 2012) (Figure 3.8 and Table 3.5). The annotated *GSDMB* promoter and exon 1 of isoform 2 were highly methylated in all tested genotypes (Berlivet et al., 2012). *GSDMA* and *CSF3* are not expressed in LCLs. Because of this, variation in expression levels of *GSDMA* and *CSF3* due to allelic effects could not be detected or excluded. However, Lluis *et al.* showed that *GSDMA* expression levels increased in cord blood

lymphocytes from babies carrying asthma-associated 17q21 alleles (Lluis et al., 2011). Additionally, a recent study found a common variant in *CSF3* that was associated with asthma (Zavbi et al., 2016). Therefore, *GSDMA* and *CSF3* should not be ruled out as candidate genes in asthma predisposition. To validate promoter methylation in the 17q21 region, we tested the methylation profiles of the *GSDMA* and *CSF3* promoter regions in LCLs and found inter-individual variation among LCLs with respect to methylation levels (Table 3.5) (Berlivet et al., 2012).

3.2.2. Sex influences DNA methylation levels at the *ZPBP2* promoter region in DNA samples from peripheral blood

To investigate if the sex bias in the genetic association of asthma in the 17q21 region could result from sex differences in DNA methylation of regulatory elements in *cis*, we looked for sex-specific variation in the DNA methylation profiles of the promoters of five genes: *ZPBP2* (two regions were tested: (a) promoter/exon 1 and (b) the rs4795397 region), *GSDMB*, *ORMDL3, GSDMA* and *CSF3*. Peripheral blood DNA samples from members of the SLSJ asthmatic familial collection were tested using the bisulfite sequencing methylation assay (Table 2.5). A pilot study was conducted to assess the promoter methylation patterns in DNA samples from three males and three females of the SLSJ asthmatic familial collection (Naumova et al., 2013).

When the difference was detected the number of samples was increased. The *GSDMB* promoter and the rs4795397 region were highly methylated with little variation between sexes, whereas the *ORMDL3* promoter was unmethylated in all tested samples (Table 3.5). *GSDMA, CSF3* and *ZPBP2* promoters showed inter-individual variation in methylation, and thus, additional individuals were tested. *CSF3* and *GSDMA* showed varied methylation levels among samples, but did not show statistically significant differences in methylation levels based on sex when the number of characterized samples was increased (Student's t-test statistics, p = 9.9E-02 for *GSDMA* and p = 2.8E-01 for *CSF3*) (Figure 3.9, 3.10 and 3.12). *ZPBP2* methylation levels suggested an effect of sex on DNA methylation levels (Figure 3.11). Methylation of the *ZPBP2* promoter was then analyzed in a total of nine females and ten males. We found lower methylation levels among males (average DNA methylation level 22 %) when compared to females (average DNA methylation level 34 %) (Student's t-test statistics, p = 1E-04) (Figure 3.12) (Naumova et al., 2013).

To validate the sex-specificity of the *ZPBP2* promoter DNA methylation results, 11 CGs homozygous for the HapA haplotype from the *ZPBP2* promoter region, all located in the transcriptional start site (TSS), were analyzed using a pyrosequencing methylation assay in Dr. Laprise's laboratory. Consistent with our findings, females showed significantly higher levels of DNA methylation in the *ZPBP2* promoter compared to males (Appendix A.2). We concluded that sex-specific differences in methylation levels in the *ZPBP2* promoter region may play a fundamental role in the sex-specific genetic association in the SLSJ asthma familial collection (Naumova et al., 2013).

Numerous studies have shown that 17q21 is associated with childhood asthma (Moffatt et al., 2007, Halapi et al., 2010, Moffatt et al., 2010). The influence of age on DNA methylation of *ZPBP2* promoter region was assessed, we expected that the DNA methylation levels of the *ZPBP2* promoter will increase with age, which may influence the role of the regulatory genetic element in asthma predisposition. For that reason, *ZPBP2* average DNA methylation levels were tested and analyzed across 11 CG sites using the pyrosequencing methylation assay in Dr. Laprise's laboratory (Appendix 3.2) (Naumova et al., 2013).

3.2.3. Association between 17q21 genes methylation levels and asthma in DNA samples from peripheral blood

Given that the *ZPBP2* promoter region was highly methylated in females and adult males, anyone could expect that asthmatic individuals have lower methylation levels in *ZPBP2* compared to non-asthmatic ones. To investigate this, we compared the average DNA methylation level on the *ZPBP2* promoter region between SLSJ asthmatics and controls. No statistically significant differences in the DNA methylation level of *ZPBP2* was detected between asthmatic and non-asthmatic individuals, nor in *GSDMA, GSDMB*, *ORMDL3* and *CSF3* in the 17q21 region. However, when *ZPBP2* and *GSDMA* methylation levels were compared between asthmatic and non-asthmatic individuals, accounting for genotypes in Dr. Laprise Laboratory, they found significant association between the *GSDMA* and *ZPBP2* DNA methylation, asthma and genotype in females (Appendix A4) (Naumova et al., 2013, Al Tuwaijri et al., 2016).

3.2.4. Genotype influences DNA methylation levels at the *ZPBP2* promoter region in DNA samples from peripheral blood

Previously, we found that genotype influences the DNA methylation level of ZPBP2 and inversely correlates with RNA abundance in LCLs (Berlivet et al., 2012). Moreover, the effect of genotype on expression of ZPBP2, GSDMB, ORMDL3, and GSDMA has been demonstrated in several studies (Moffatt et al., 2007, Verlaan et al., 2009, Murphy et al., 2010, Lluis et al., 2011). Hence, the relationship between genotype and DNA methylation was tested on those genes as pilot study in DNA samples from the peripheral blood of subjects from the SLSJ asthma familial collection. For all samples, the ORMDL3 promoter was hypomethylated while the GSDMB promoter was hypermethylated, with no evidence of the effects of genotype on methylation (Table 3.5). Methylation of the ZPBP2 promoter varied considerably among individuals. As a rule, the region selected for methylation analysis should contain at least one SNP if possible (Table 2.7). ZPBP2 methylation levels were analyzed in nine homozygous HapA individuals, six heterozygous HapAB individuals, and four homozygous HapB individuals by using sodium bisulfite methylation analysis. We did not observe an effect of genotype on ZPBP2 methylation level, and the average DNA methylation level was HapA = 29%, HapAB = 27%, and HapB = 27%. Subsequently, we separately tested the influence of genotype on the ZPBP2 methylation level in males and females. We did not find statistically significant effects of genotype on the ZPBP2 methylation level in males or females. However, when the number of samples was increased to analyze the methylation level of nine CGs from the ZPBP2 promoter region overlapping the TSS, which included 37 homozygous HapA, 60 heterozygous HapAB, and 60 homozygous HapB samples using the pyrosequencing methylation assay in Dr. Laprise's Laboratory. We noticed correlations between all nine CGs using Pearson's correlation tests (correlation coefficients between 0.6 and 1.0), hence, the average methylation levels for all the nine CGs was analyzed. As we previously showed that there is association between ZPBP2 methylation levels and the sex of individuals in HapA homozygotes (Naumova et al., 2013). The methylation levels were analyzed in the other two genotypic groups HapB and HapAB and showed that the methylation levels were increased in females compared to males (HapB $p = 3.3E-04$ and HapAB $p = 9.0E-10$, Student's t-test), consistent with our previous findings as well as with the findings of others (Al Tuwaijri et al., 2016, McCarthy et al., 2014, Yousefi et al., 2015, Naumova et al., 2013). Additionally, we observed that when the effect of genotype on methylation was separately analyzed in each sex, six

out of nine tested CGs were hypermethylated in homozygous HapB individuals (p-values ranging from 7.7E-04 to 5.1E-09 for females and ranging from 7.3E-09 to 5.1E-09 for males) (Table 3.6) (Al Tuwaijri et al., 2016).

3.2.5. Genotype influences DNA methylation levels at the *GSDMA* promoter region in DNA samples from peripheral blood

The *GSDMA* promoter showed variable DNA methylation levels in DNA samples from peripheral blood. We found a statistically significant allelic effect on the *GSDMA* methylation level, while the rs3902025-T allele (risk allele for asthma) (n=6) had a lower methylation level compared to the G non-risk allele (n=4) (p =1.13E-02). The significance of the haplotype effect was lost after correction for multiple testing (p value significance threshold after correction is 1.0E-02). Therefore, the sample size was increased then we tested whether inter-individual variation in methylation levels at *GSDMA* promoters depended on genotype by using pyrosequencing methylation assays in Dr. Laprise's laboratory. We analyzed the DNA methylation levels for three CGs from the *GSDMA* promoter region, all located upstream of the TSS, in 17 homozygous HapA, 60 heterozygous and 13 homozygous HapB individuals (Table 3.7). When correlation between methylation levels at individual CGs was tested, all the three CGs showed significant correlation, Pearson's correlation coefficient ranging from (0.5 and 0.8), then the average methylation levels was analyzed through the region (Al Tuwaijri et al., 2016). Analysis of DNA methylation levels in all three CGs in homozygous samples showed the influence of genotype. Additionally, we noticed no significant differences between the methylation levels in heterozygous samples and those of HapA homozygous samples (Al Tuwaijri et al., 2016) (Table 3.7). We concluded that DNA methylation levels of *ZPBP2* and *GSDMA* promoters in peripheral blood cells depend on genotype. For both genes, HapB alleles show higher methylation levels in homozygous individuals.

3.2.6. Treatment with DNA methyltransferase 1 inhibitor 5-aza-dC caused changes in genes expression in the 17q21 region

We determined that local genotype influences the methylation level of the promoter regions of *GSDMA* and *ZPBP2*. We then asked whether changes in DNA methylation influence expression levels of genes *in vitro* in 17q21. HEK293T/17 and NuLi-1 cells were treated with 5-aza-dC to

induce demethylation as previously described (for more details regarding optimization of 5-aza-dC concentration, refer to (Figure 3.6A and B)). In both cell lines, cell apoptosis was clearly identified after treatment with 5-aza-dC, also we noticed increase in *H19* expression 11- and 5-fold in HEK293T/17 and NuLi-1, respectively (Figure 3.13A and B). *GSDMA* expression levels were upregulated in both cell lines (6.2-fold in HEK293T/17 and 4.2-fold in NuLi-1). *ORMDL3* expression levels were slightly increased in HEK293T/17 cells by 1.4-fold. However, NuLi-1 did not show a significant change in *ORMDL3* expression. *ZPBP2* was not expressed in HEK293T/17 before treatment and remained silent after treatment, while in NuLi-1, *ZPBP2* expression levels showed a highly significant increase (20-fold) following 5-aza-dC treatment. We found that *IKZF3* was expressed in both cell lines and the expression was increased only in HEK293T/17 cells following 5-aza-dC treatment. Furthermore, in NuLi-1 cells, we noticed significant upregulation of *GSDMB* expression (4-fold), whereas in HEK293T/17, expression of *GSDMB* was very modest (Figure 3.13B). NuLi-1 was a suitable cell line to assess allelic expression analysis before and after 5-aza-dC treatment, as it is the only one that is heterozygous for the region between *IKZF3* and *GSDMA* while still expressing all five genes in the 17q21 region. Contrastingly, the HEK293T/17 cell line is homozygous, therefore it is inconclusive in this case (Table 2.3). In control NuLi-1 cells, *IKZF3, ZPBP2*, *ORMDL3* and *GSDMA* all showed allelic bias in expression, *IKZF3* and *ZPBP2* were preferentially expressed toward the HapB alleles, but *ORMDL3* and *GSDMA* were preferentially expressed toward the HapA alleles, while *GSDMB* was expressed biallelically (Figure 3.13C). After 5-aza-dC treatment, we noticed that the asthma-associated HapA allele of *ZPBP2* was reactivated, although the stronger expression was still from the HapB allele. We noticed that the allele-specific difference of *ORMDL3* gene expression was switched toward the HapB instead of the HapA allele following 5-aza-dC treatment. Both *GSDMA* and *GSDMB* were upregulated after NuLi-1 was treated with 5-aza-dC, although there were no changes in allelic expression (Figure 3.13C) (Moussette et al., 2017).

3.2.7. Alterations in DNA methylation at *ZPBP2* and *GSDMA* promoters post 5-aza-dC treatment

Influence of 5-aza-dC treatment on expression levels of genes in 17q21 was mainly noticeable in *ZPBP2* in NuLi-1 and *GSDMA* in HEK293T/17 and NuLi-1 cell lines. Then we asked whether demethylation of *ZPBP2* and *GSDMA* has a role in the increase of the *ZPBP2* and *GSDMA*

expression. To answer this question, methylation profiles of these genes were assessed by a sodium bisulfite sequencing methylation assays in NuLi-1 and HEK293T/17 cells. (the methylation assays and analysis in NuLi-1 was conducted by S. Moussette) (Moussette et al., 2017). When NuLi-1 cells were treated with 5-aza-dC, we indicated decrease in DNA methylation levels in the promoter region of *ZPBP2* using the sodium bisulfite sequencing methylation assay, the reduction was from (16% to 5%) (Figure 3.14A), this was in agreement with the pyrosequencing methylation assay results, four out of eleven CGs showed significant reduction in *ZPBP2* DNA methylation levels (table A.1). Treatment HEK293T/17 cells with 5-aza-dC did not change the highly-methylated levels of *ZPBP2* promoter region (control 64.3% compared to treated cells 62.3%). We noticed decrease in the DNA methylation levels of *GSDMA* promoter region, (control 57% compared to treated cells 25%) in NuLi-1 (Figure 3.14B), and (control 74% compared to treated cells 61%) in HEK293T/17 cells after 5-aza-dC treatment. Which indicated that the small reduction in *ZPBP2* and *GSDMA* promoter methylation levels has remarkable influences in their expression (Moussette et al., 2017).

We asked if changes in *ZPBP2* and *GSDMA* allelic bias in NuLi-1 cells was caused by demethylation, allelic methylation levels were assessed in both genes. We noticed that in NuLi-1 controls and 5-aza-dC treated cells, the HapA allele of the *ZPBP2* promoter was more methylated than the HapB allele (20% compared to 10% methylation in controls, and 8% compared to 2% in 5-aza-dC treated cells, respectively) (Figure 3.14A). This correlated with the expression bias towards the HapB allele. When we analyzed the average percentage of methylation for each individual CG, we found 5 CGs (31, 40, 41, 48, 51) that showed a major bias between alleles, and the most pronounced one that showed clear reduce in methylation levels following 5-aza-dC was in CG31 that located proximal to *ZPBP2* TSS, (Figure 3.14A and Table A.1). The difference in CG31 methylation levels on the HapA allele (control 88% compared to treated cells 36%) suggests the possible involvement of CG31 in increasing *ZPBP2* expression in this allele following 5-aza-dC treatment. In the *GSDMA* promoter region, the HapA allele showed higher methylation levels than the HapB allele in both controls and 5-aza-dC treated NuLi-1 cells (65% compared 49% methylation in controls, and 27% compared 24% in treated cells, respectively) (Figure 3.14B). This indicated that the allelic methylation levels of *GSDMA* promoter did not correlate with allelic expression bias which was in favor of the HapA. We also assessed whether differences in the allelic expression of *ORMDL3* were due to promoter demethylation. We found that the methylation

levels of *ORMDL3* promoter was very low in controls and 5-aza-dC treated NuLi-1 cells (Figure 3.14C), suggesting that promoter methylation did not explain the allelic imbalance found in the expression of both *GSDMA* and *ORMDL3.* Instead, there is a possibility that this bias results from alteration in DNA methylation at another regulatory region such as enhancers or insulators (Moussette et al., 2017).

3.3. X chromosome dosage and presence of sex-determining region Y (*SRY*) may cause epigenetic sex differences in asthma region 17q21

3.3.1. Sex chromosome dosage and presence of *SRY*, but not sex phenotype, influences sex differences in DNA methylation levels in *ZPBP2* promoter /exon1

We found that DNA methylation is significantly higher in females than males at the *ZPBP2* promoter region in DNA from PBCs (Naumova et al., 2013). To understand the potential mechanism that causes sex differences in methylation levels at the *ZPBP2* promoter, DNA methylation was tested in DNA samples extracted from 21 untransformed human fibroblast cell lines derived from individuals with different sex chromosome dosage: 46, XY females (sex reversal); 45, X females (Turner syndrome); 46, XY males; 46, XX females; 46, XX males; and a 49, XXXXY male (Table 2.4). The influence of sex phenotype, presence or absence of the sex determining region Y (*SRY*), presence or absence of Y chromosomes, and the X-chromosome copy number (one copy *vs.* more than one X copy) on *ZPBP2* DNA methylation levels was analyzed. All fibroblast cell lines were genotyped for the *SRY* gene (Table 3.8). If sex chromosome dosage influences *ZPBP2* DNA methylation levels, we would expect to detect a direct correlation between X or Y chromosome dosage and higher *ZPBP2* DNA methylation levels regardless of sex phenotype. If sex phenotype affects DNA methylation levels at the *ZPBP2* region, then we would expect to detect similar *ZPBP2* DNA methylation levels between members of each sex, regardless of how many X chromosomes each member carries. (Table 3.12 contains a detailed explanation for the *ZPBP2* DNA methylation expectation). No statistically significant differences were found between sex phenotype, samples with and without the *SRY* gene, and presence or absence of Y chromosome and *ZPBP2* DNA methylation level (Student's t-test statistics, p = 2.25E-01, p = 8.66E-02 and p = 1.1E-01 respectively) (Figure 3.15A, B). However, a significant positive correlation was found between *ZPBP2* methylation and the X-chromosome copy number. There

was a considerably lower methylation levels among individuals with one X copy (n = 9, average DNA methylation level 18%) when compared to individuals with more than one X copy (n = 7, average DNA methylation level 29%) (Student's t-test statistics, p = 2.30E-03) (Figure 3.15C). Samples were analyzed with respect to both X chromosome copy and presence or absence of *SRY*, and we noticed that samples with more than one X chromosome copy, in the absence of *SRY*, have significantly higher DNA methylation levels of *ZPBP2* (p = 5.4E-02; one-way ANOVA) (Figure 3.15D). This finding suggests that *SRY* may interacts with *ZPBP2* promoter/exon1 and play protective roles against *ZPBP2* DNA methylation. There are several X-linked genes whose main functions are to contribute to epigenetic regulatory mechanisms, and they may be involved in sex bias in DNA methylation. The effect of mutations on methyl-CpG-binding protein 2 (MECP2) in *ZPBP2* DNA methylation status was analyzed. The selection of *MECP2* was based on its function as an X-linked gene that recognizes methylated DNA via its methyl-CpG–binding domain (MBD) (Hansen et al., 2010). In addition, *MeCP2* is considered a methylation-specific transcriptional repressor that interacts with chromatin-modifying complexes, histone Deacetylase **(**HDAC) and sin3A, to repress transcription (Jones et al., 1998). *ZPBP2* DNA methylation was tested in DNA samples extracted from three fibroblast cell lines of Rett syndrome (RTT) patients. RTT is a neurodevelopmental disorder that affects girls and is caused by a mutation *MeCP2* (Nan et al., 1998). Although patients with RTT carry two X chromosomes, we noticed a reduction in their *ZPBP2* methylation level, which were comparable with the methylation levels of samples with one X chromosome (46,XY males and 46,XY sex reversal) (20% in Rett syndrome samples *vs* 34% in 46,XX male syndrome, p = 5.0E-02, Student's t-test). This suggests that *MeCP2* could be a good candidate gene that may be involved in sex bias in DNA methylation, although this theory needs to be investigated in larger sample sizes (Figure. 3.16).

3.3.2. DNA methylation levels of *ZPBP2* are significantly correlated with the age of cell line donors in fibroblast cell lines

Cell lines with more than one copy of the X chromosome have a significantly higher methylation level in *ZPBP2* than those with only one X. However, we noticed a variable distribution in methylation levels within fibroblast samples containing more than one X chromosome copy (from 18% to 46%). We sought to determine the cause(s) of this variability in methylation levels within more than one X group, since extended cell culture and the age of cell

line donors may alter DNA methylation patterns (Saferali et al., 2010) (Grafodatskaya et al., 2010, Mays-Hoopes, 1989, Jung and Pfeifer, 2015). Pearson's correlation analyses indicated no correlation between DNA methylation of *ZPBP2* and the number of cell line passages in individuals carrying one X and more than one X. However, there was a significant correlation between DNA methylation of *ZPBP2* and the age of cell line donors who carried more than one X chromosome (Pearson correlation coefficient 0.5).

3.3.3. Knocking down the X-linked *KDM5C* gene in the HEK293T/17 cell line did not significantly affect *ZPBP2* promoter methylation levels

Kdm5c showed female sex bias in gene expression in mice brains that depended on the sex chromosome dosage rather than the gonadal sex of the mice (Xu et al., 2002) (Arnold et al., 2012). We started a pilot experiment using a candidate gene approach to test the possibility that *KDM5C* plays a role in the maintenance of sex-specific differences in methylation levels at the *ZPBP2* promoter. HEK293T/17 was transfected by siKDM5C that targeted four different exons in *KDM5C* (Figure 2.1). The specificity of the siKDM5C construct was confirmed by measuring the mRNA level of *KDM5C* in HEK293T/17 transfected separately with siKDM5C, small interfering CCCTC-binding factor (siCTCF), and small interfering P300 (siP300) (n = 2). *KDM5C* expression was reduced 2.7-fold only in HEK293T/17 transfected with siKDM5C, indicating that siKDM5C is targeting specifically *KDM5C* (Figure 3.17A). We expected that the depletion of the X-linked *KDM5C* gene in HEK293T/17 would decrease the DNA methylation at the *ZPBP2* promoter. After 72 h of HEK293T/17 transfection with siKDM5C to reduce the expression of *KDM5C* in HEK293T/17 cells, the RNA was extracted. RT-PCR was applied to evaluate the expression level of *KDM5C*. Results showed about 75% depletion in *KDM5C* expression (n = 3) (Figure.3.17B). *DNMT1* is responsible for the maintenance DNA methylation during replication and copying the methylation patterns to the daughter strands (Hermann et al., 2004). Knocking down the X-linked *KDM5C* that encodes a histone demethylase, which removes di- and tri-methylation of histone H3 lysine 4, affects the *KDM5C* transcriptional repression activity (Outchkourov et al., 2013) which in turn may influence *DNMT1* expression. It has been reported that *DNMT3A* contains an ATRX–DNMT3–DNMT3L (ADD) domain that has been found to interact with histone H3 unmethylated at K4, while H3K4me2 and H3K4me3 inhibit this interaction (Otani et al., 2009). To understand how knocking down *KDM5C* may affect *DNMT3A* and *DNMT1*, the expression of both was

assessed and showed no change (n = 3) (Figure.3.17B). It has been demonstrated that DNA methylation differences were associated with the *KDM5C* mutation at specific loci at CGIs (Grafodatskaya et al., 2013). Also, females have significantly higher levels of DNA methylation than males in multiple autosomal gene promoters, so we chose to assess the expression level of the top three candidate genes they indicated, including *CACYBP*, *FBXL5*, and *SCMH1* (Grafodatskaya et al., 2013). The expression levels of these three genes in the transfected HEK293T/17 with siKDM5C were tested. Our expectation was that reduction of the *KDM5C* RNA led to an increase in expression levels of these genes. No significant difference in expression level of *CACYBP*, *FBXL5* and *SCMH1* has been found between control and *KDM5C* knock down HEK293T/17 (n = 3) (Figure.3.17B). We then asked if any of the genes in the 17q21 asthma-associated region were affected by *KDM5C* knockdown, and we tested the expression level of *ZPBP2* adjacent genes: *IKZF3*, *ORMDL3*, and *GSDMB*. *IKZF3* showed a 2-fold increase in expression following siKDM5C treatment in HEK293T/17 (n = 3), while *ORMDL3* and *GSDMB* did not show any significant change in their expression levels (Figure.3.17B). Knocking down *KDM5C* in HEK293T/17 did not affect the maintenance of sex-specific differences in methylation levels at the *ZPBP2* promoter, whereas the expression levels of the *ZPBP2*-neighboring gene *IKZF3* was up-regulated, suggesting that *ZPBP2* promoter harbors an enhancer (or a cluster of enhancers) that may control expression of the distally located gene.

DNA methylation level of the *ZPBP2* promoter/exon1 region was assessed in the transfected HEK293T/17 with siKDM5C and control using a sodium bisulfate methylation assay. We expected to detect a decrease in the methylation levels of the *ZPBP2* promoter region in the siKDM5C transfected cell line compared to the control. No change in DNA methylation level at *ZPBP2* promoter region was detected between the control and the transfected HEK293T/17. The average DNA methylation of *ZPBP2* was (96% and 97%), respectively (Figure 3.18A). This result was validated in Dr. Laprise's laboratory using pyrosequencing methylation assays and showed the same results (Figure 3.18B).

TABLES

Table 3.1 Summary of DNA methylation analysis of eight promoter regions in 5q31 in lymphoblastoid cell lines.

Gene	*Assayed region Genome coordinates Chr.5 (hg19)*	*CG island*	*Number of CGs tested*	*Number of LCLs tested*	*Average methylation in percent (range)*	*Effect of genotype on average methylation p value Student's t-test*	*Effect of sex on average methylation p value Student's t-test*
IL3	131,396,291-131,396,592	No	9	7	71 (42-85)	1.66E-01	5.56E-01
CSF2	131,409,461-131,409,829	No	7	8	79 (41-96)	1.06E-01	4.96E-01
P4HA2	131,562,728-131,563,038	No	11	7	51 (37-67)	**3.06E-02**	6.93E-01
PDLIM4	131,592,816-131,593,188	Yes	24	7	41 (26-55)	4.40E-01	5.25E-01
SLC22A4	131,630,179-131,630,650	Yes	42	8	1 (0-3)	8.26E-01	2.16E-01
SLC22A5	131,705,744-131,706,217	Yes	39	8	9 (2-18)	1.88E-01	6.39E-01
IRF1	131,826,332-131,826,827	Yes	49	6	0.3 (0-0.6)	6.77E-01	2.91E-01
IL5	131,878,921-131,879,466	No	7	8	62 (44-75)	1.05E-01	7.56E-01

Table 3.2 Effect genotype on DNA methylation at the *P4HA2* in the promoter region in DNA samples from lymphoblastoid cell lines.

CG	***Average methylation in percent***		***p-value***
	HapC	HapD	
CG01	82	58	1.74E-01
CG02	91	79	2.71E-01
CG03	62	97	**1.10E-03**
CG04	74	72	9.17E-01
CG05	59	98	2.33E-01
CG06	19	58	1.73E-02
CG07	17	49	1.55E-01
CG08	0	40	1.17E-01
CG09	17	44	1.30E-01
CG10	5	14	5.74E-01
CG11	24	21	8.52E-01
Average	41	57	**3.06E-02**

The numbers in the cells represent the p values from Student's t-test statistics. Due to the number of tests, correction for multiple testing set the significance level at p = 0.05/11 = 4.5E-03. Significant P values are shown in bold.

Table 3.3 Effect genotype on DNA methylation at the *SLC22A5* in the promoter region in DNA samples from lymphoblastoid cell lines.

CG	*Average methylation in percent*		*p-value*	*CG*	*Average methylation in percent*		*p-value*	*CG*	*Average methylation in percent*		*p-value*
	Females	**Males**			**Females**	**Males**			**Females**	**Males**	
CG01	3	4	5.76E-01	**CG14**	1	0	3.56E-01	**CG27**	2	4	7.41E-01
CG02	2	2	9.39E-01	**CG15**	1	0	3.56E-01	**CG28**	1	1	9.53E-01
CG03	1	1	9.53E-01	**CG16**	1	0	3.56E-01	**CG29**	9	11	8.94E-01
CG04	0	2	1.34E-01	**CG17**	0	0	-	**CG30**	16	2	2.00E-01
CG05	0	4	**8.00E-07**	**CG18**	1	2	5.34E-01	**CG31**	1	0	3.56E-01
CG06	2	8	4.43E-01	**CG19**	0	1	3.56E-01	**CG32**	9	11	6.93E-01
CG07	2	8	4.24E-01	**CG20**	0	1	3.56E-01	**CG33**	45	25	3.68E-01
CG08	1	1	9.28E-01	**CG21**	1	1	9.77E-01	**CG34**	53	5	1.11E-01
CG09	0	0	-	**CG22**	1	1	9.28E-01	**CG35**	35	13	2.98E-01
CG10	2	2	9.86E-01	**CG23**	1	3	2.19E-01	**CG36**	42	32	7.30E-01
CG11	3	0	1.64E-01	**CG24**	0	4	1.85E-01	**CG37**	22	31	7.03E-01
CG12	0	0	-	**CG25**	8	3	5.69E-01	**CG38**	42	47	8.09E-01
CG13	0	0	-	**CG26**	6	8	7.22E-01	**CG39**	52	56	8.74E-01

The numbers in the cells represent the p values from Student's t-test statistics. Due to the number of tests, correction for multiple testing set the significance level at p = 0.05/39 = 1.2E-03. Significant P values are shown in bold.

Table 3.4 Effect genotype on DNA methylation at the *SLC22A5* CG-island shore in the promoter region in DNA samples from human peripheral blood cells of the Saguenay-Lac-Saint-Jean asthma familial collection.

CG	***Average methylation in percent***			***p-value***		
	HapC	**HapCD**	**HapD**	**HapC vs HapD**	**HapC vs HapCD**	**HapD vs HapCD**
CG31	7	8	14	**6.3E-05**	1.9E-01	**2.2E-12**
CG32	14	23	26	**7.8E-07**	**9.7E-04**	1.0E-01
CG33	24	51	43	**4.2E-10**	**1.3E-08**	**6.5E-04**
CG34	15	24	26	**1.2E-05**	**2.5E-04**	3.5E-01
CG35	28	45	49	**2.7E-06**	**1.3E-04**	8.5E-02
CG36	26	34	40	**1.5E-05**	6.1E-03	**8.4E-05**
CG37	38	44	57	**1.1E-07**	1.4E-02	**6.0E-12**
Average	22	33	36	**2E-06**	**1E-03**	5E-03

The numbers in the cells represent the p values from Student's t-test statistics. Due to the number of tests, correction for multiple testing set the significance level at p = 0.05/21 = 2.4E-03. Significant P values are shown in bold.

Table 3.5 Summary of DNA methylation analysis of seven 17q21 regulatory regions in the LCLs and peripheral blood DNA from individuals of the Saguenay-Lac-Saint-Jean asthma familial collection.

Genomic region	*Number of CGs in tested region*	*Number of LCLs tested by the candidate*	*Number of PCR replicates*	*Number of asthmatic and non-asthmatic peripheral blood DNA tested by the candidate*	*Average number of clones analyzed*	*Number of samples analyzed by other members of the lab*	*Whole blood average methylation in percent (range)*	*Number of samples that was used in the analysis*	*DNA methylation varies with genotype of individuals*	*DNA methylation varies with age of individuals*	*DNA methylation varies with sex of individuals*
IKZF3	43	5	2	0	15	-	1 (0.2-1)	5	No	No	No
***ZPBP2* promoter, rs4795397 region**	3	2	2	6	22	-	86 (72-97)	6	No	No	No
ZPBP2	51	0	2	19	15	15	27 (19-40)	15	Yes	Yes	Yes
GSDMB	5	2	2	6	21	-	96 (91-98)	6	No	No	No
ORMDL3	32	0	2	6	15	-	0 (0-1)	6	No	No	No
GSDMA	5	4	2	9	21	-	63 (50-78)	9	Yes	No	No
CSF3	6	2	2	10	22	-	35 (27-43)	10	No	No	No

Table 3.6 Effect of genotype on DNA methylation at the *ZPBP2* promoter region in human peripheral blood cells from SLSJ collection.

CG	*Average/ median methylation in percent (Females)*			*Average/ median methylation in percent (Males)*			*P value females*			*P value males*		
	HapA n=19	**HapA B n=30**	**HapB n=30**	**HapA n=18**	**HapAB n=30**	**HapB n=30**	**HapA vs HapB**	**HapA vs HapAB**	**HapB vs HapAB**	**HapA vs HapB**	**HapA vs HapAB**	**HapB vs HapAB**
CG1	34/34	31/30	42/40	32/22	25/23	39/38	**7.7E-04**	3.3E-01	**2.1E-07**	**7.3E-09**	5.6E-01	**8.7E-09**
CG2	41/41	39/39	54/54	28/27	33/32	51/50	**4.4E-07**	8.2E-01	**5.9E-09**	**5.1E-09**	9.8E-02	**5.1E-09**
CG3	27/27	28/28	38/38	20/20	24/23	35/34	**5.1E-09**	5.7E-01	**5.1E-09**	**5.1E-09**	1.4E-03	**5.1E-09**
CG4	33/32	31/30	35/35	24/24	23/22	27/27	5.3E-01	3.4E-01	1.6E-02	4.1E-03	4.9E-01	**6.0E-06**
CG7	34/34	31/30	32/32	26/26	23/22	27/27	1.0E-01	1.3E-02	6.1E-01	9.5E-01	3.0E-03	**1.6E-04**
CG8	34/34	30/30	33/33	27/27	24/24	29/29	7.4E-01	2.3E-02	2.3E-02	8.2E-02	5.3E-03	**7.8E-08**
CG9	32/32	32/32	39/38	26/25	24/24	34/34	**7.0E-06**	9.4E-01	**9.3E-08**	**5.1E-09**	3.7E-01	**5.1E-09**
CG10	24/24	22/22	33/33	18/18	16/16	2828	**6.8E-09**	4.2E-01	**5.1E-09**	**5.1E-09**	9.2E-02	**5.1E-09**
CG11	35/35	31/31	49/49	27/26	25/24	45/45	**5.2E-09**	4.7E-02	**5.1E-09**	**5.1E-09**	4.4E-01	**5.1E-09**
Average	35/32	31/31	39/39	24/24	24/24	35/35	**5.0E-06**	3.1E-01	**5.4E-09**	**5.1E-09**	9.9E-01	**5.1E-09**

The numbers in the cells represent the p values from Student's t-test statistics. Due to the number of tests, correction for multiple testing set the significance level at p = 0.05/54 = 9.3E-04. Significant P values are shown in bold.

Table 3.7 Effect of genotype on DNA methylation at the *GSDMA* promoter region in DNA samples from human peripheral blood cells from the SLSJ collection.

	Average/median Methylation in percent			***p-value***		
CG	**HapA (n=17)**	**HapAB (n=60)**	**HapB (n=13)**	**HapA vs HapB**	**HapA vs HapAB**	**HapB vs HapAB**
CG1	19/19	19/19	25/24	**2.0E-06**	7.4E-01	**3.6E-07**
CG2	69/69	69/68	78/77	**3.3E-05**	9.9E-01	**1.0E-06**
CG3	71/70	70/69	79/78	**1.0E-03**	9.5E-01	**2.3E-05**
Average	53	53	60	**6.0E-06**	9.9E-01	**1.1E-07**

The numbers in the cells represent the p values from Student's t-test statistics. Due to the number of tests, correction for multiple testing set the significance level at p = 0.05/9 = 5.5E-03. Significant P values are shown in bold.

Table 3.8 Summary of *ZPBP2* DNA methylation analysis in fibroblast samples from individuals with different sex chromosome dosage were analyzed by bisulfite sequencing methylation analysis. All fibroblast samples were genotyped for *SRY* gene by conventional PCR. Number of Xq chromosome information from https://catalog.coriell.org.

Fibroblast sample description	***Coriell ID***	***SRY PCR***	***Number of Xq chromosome***	***Percent of DNA Methylation for ZPBP2 using Sodium bisulfite sequencing methylation assays***
NORMAL MALE	GM02936	M	1	12
NORMAL MALE	GM03348	M	1	23
NORMAL MALE	GM07753	M	1	18
NORMAL FEMALE	GM00037	F	2	37
NORMAL FEMALE	GM01652	F	2	20
NORMAL FEMALE	GM00038	F	2	18
TURNER SYNDROME	GM00857	F	3	39
TURNER SYNDROME	GM02668	F	1	18
TURNER SYNDROME	GM00735	M	1	19
TURNER SYNDROME	GM01176	F	3	27
TURNER SYNDROME	GM00088	F	1	14
XXXY AND XXXXY SYNDROME	GM00157	M	3	30
46,XY SEX REVERSAL SRY	GM00048	F	1	19

46,XY SEX REVERSAL SRY	GM01628	F	1	21
46,XY SEX REVERSAL SRY	GM03368	F	1	14
XX MALE SYNDROME	GM02670	M	2	25
XX MALE SYNDROME	GM02626	M	2	30
XX MALE SYNDROME	GM01889	M	2	46
RETT SYNDROME; RTT	GM07982	F	2	18
RETT SYNDROME; RTT	GM11271	F	2	24
RETT SYNDROME; RTT	GM11273	F	2	19

Table 3.9 Expectation of *ZPBP2* DNA methylation level after performing sodium bisulfite sequencing methylation assays using different sex and karyotype combinations of fibroblast cell lines (Sex phenotype information from https://catalog.coriell.org). Fibroblast cell lines description is listed in (Table 2.4).

	46, XX Female	***46, XX Male***	***45, X Female***	***46, XY Male***	***46, XY Female***	***49, XXXXY Male***
X chromosome dosage influences Sex-specific DNA methylation differences levels at the ZPBP2	High	High	Low	Low	Low	Highest
Y chromosome dosage influences Sex-specific DNA methylation differences levels at the ZPBP2	High	High	High	Low	Low	Low
Sex phenotype influences Sex-specific DNA methylation differences levels at the ZPBP2	High	Low	High	Low	High	Low

FIGURES

Figure 3.1 *SLC22A4* exon 1 methylation patterns in LCLs. **A.** Location of the interrogated CGs with respect to *SLC22A4* promoter, exon 1 and CG island. All features are shown in the context of the UCSC genome browser, assembly GRCh37/hg19 (http://genome.ucsc.edu). **B.** *SLC22A4* methylation. Filled circles represent methylated cytosines, open circles represent unmethylated cytosines in CG pairs. Each row represents the methylation pattern of a single clone, i.e. one allele. LCL ID numbers are shown on the left. The percent on the left represents the average percent of methylation. The left three panels show results for HapC homozygous LCLs, the right three panels show results for HapD homozygous LCLs.

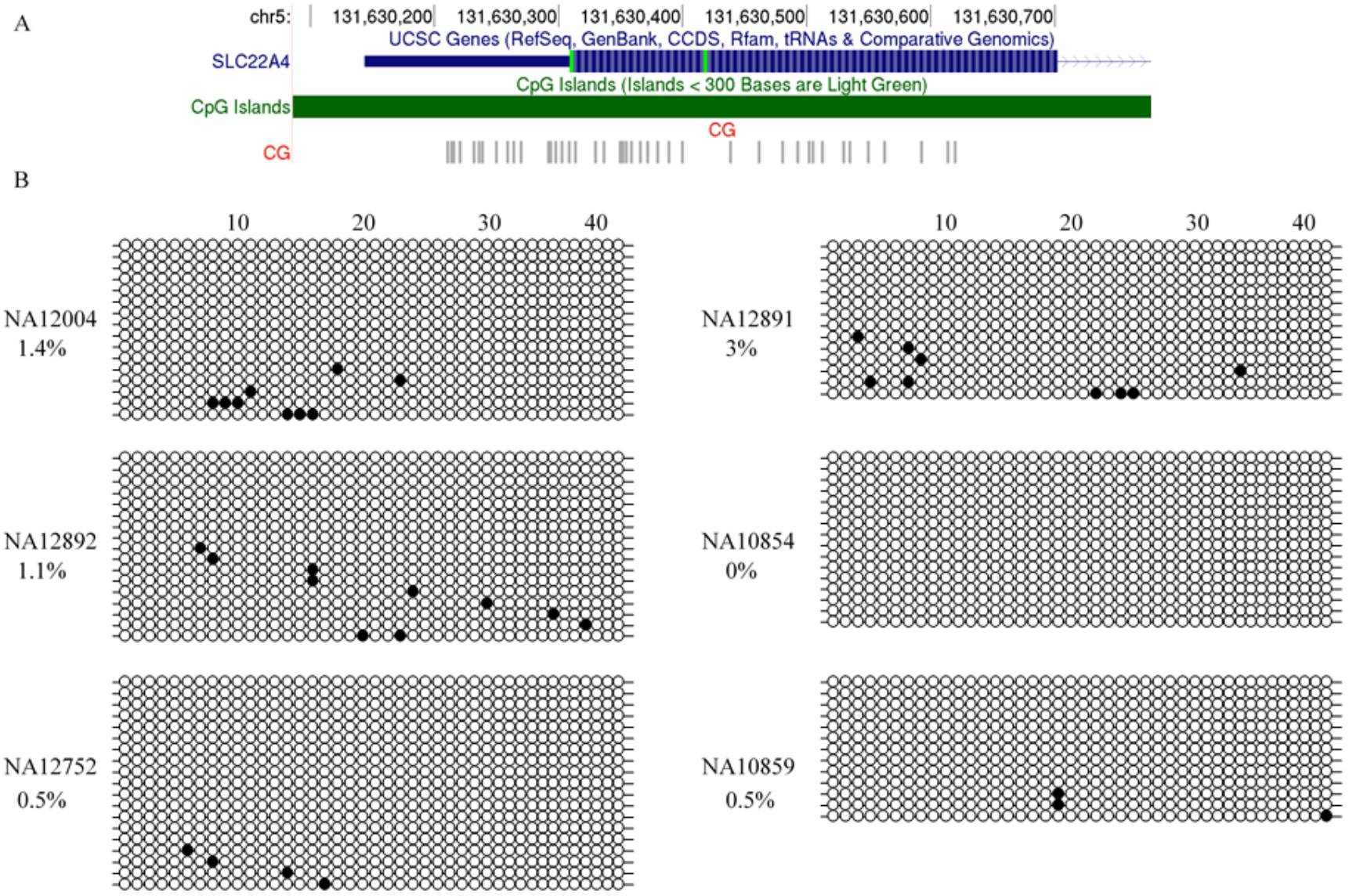
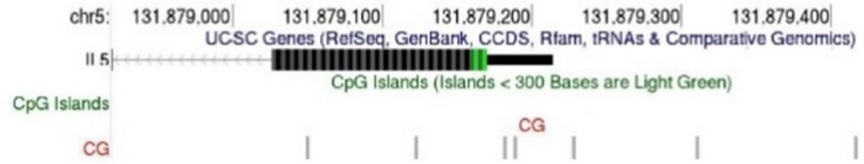

A
chr5:
131,630,200
131,630,300
131,630,400
131,630,500
131,630,600
131,630,700
UCSC Genes (RefSeq, GenBank, CCDS, Rfam, tRNAs & Comparative Genomics)
SLC22A4
CpG Islands (Islands < 300 Bases are Light Green)
CpG Islands
CG
CG
B
10
20
30
40
NA12004
1.4%
NA12891
3%
NA12892
1.1%
NA10854
0%
NA12752
0.5%
NA10859
0.5%

Figure 3.2 *IL5* promoter/exon 1 DNA methylation patterns in LCLs. **A**. Location of the interrogated CGs with respect to *IL5* promoter and exon 1. All features are shown in the context of the UCSC genome browser, assembly GRCh37/hg19 (http://genome.ucsc.edu). **B.** *IL5* methylation. Filled circles represent methylated cytosines, open circles represent unmethylated cytosines in CG pairs. Each row represents the methylation pattern of a single clone, i.e. one allele. IDs of LCLs are shown on the left. The percent on the left represents the average percent methylation for the LCL. The number of clones with the same methylation profile is shown on the right. The left three panels show results for HapC homozygous LCLs, the right three panels show results for HapD homozygous LCLs.

A


chr5: 131,879,000 131,879,100 131,879,200 131,879,300 131,879,400
UCSC Genes (RefSeq, GenBank, CCDS, Rfam, tRNAs & Comparative Genomics)
Il 5
CpG Islands (Islands < 300 Bases are Light Green)
CpG Islands
CG
CG

B

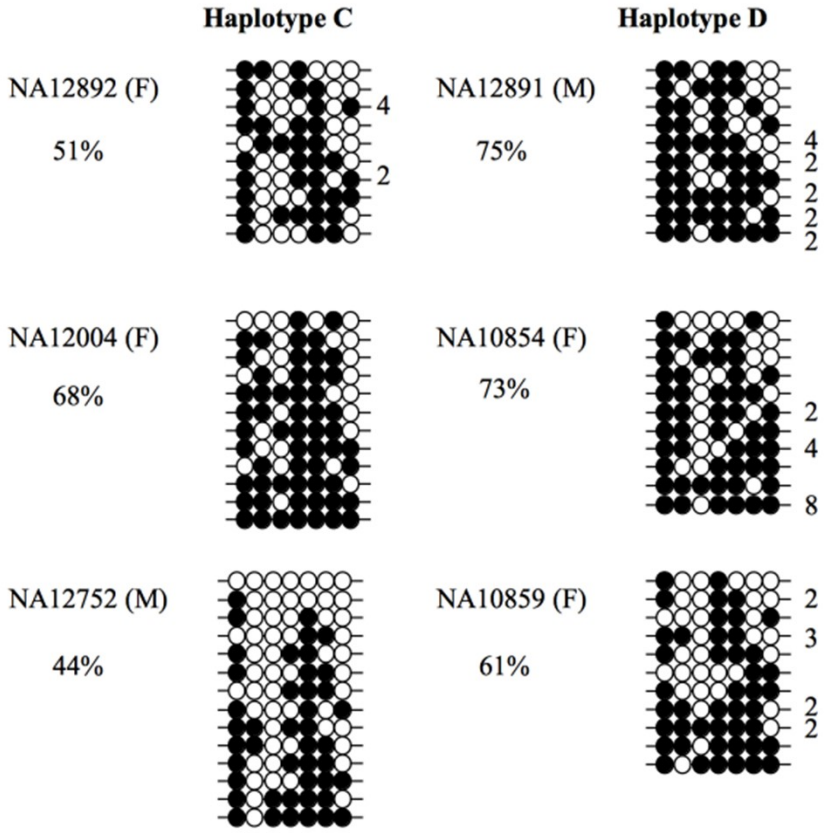

Haplotype C
Haplotype D
NA12892 (F)
51%
4
2
NA12891 (M)
75%
4
2
2
2
2
NA12004 (F)
68%
NA10854 (F)
73%
2
4
8
NA12752 (M)
44%
NA10859 (F)
61%
2
3
2
2

Figure 3.3 *IL3* promoter/exon 1 DNA methylation profiles in LCLs. **A.** Location of the interrogated CGs with respect to *IL3* promoter and exon 1. All features are shown in the context of the UCSC genome browser, assembly GRCh37/hg19 (http://genome.ucsc.edu). **B.** For symbol descriptions please see figure 3.2.

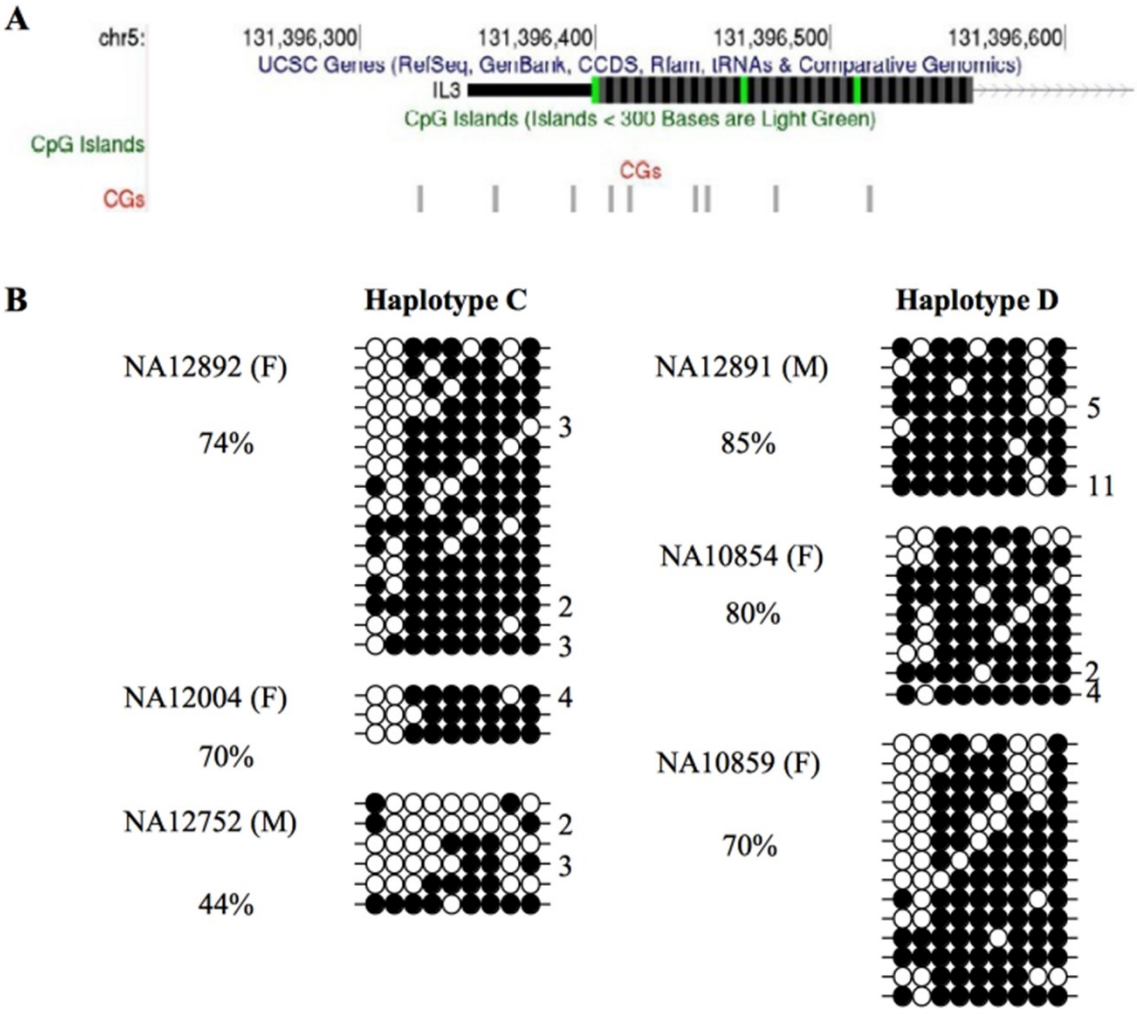

A
chr5:
131,396,300
131,396,400
131,396,500
131,396,600
UCSC Genes (RefSeq, GenBank, CCDS, Rfam, tRNAs & Comparative Genomics)
IL3
CpG Islands (Islands < 300 Bases are Light Green)
CpG Islands
CGs
CGs
B
Haplotype C
Haplotype D
NA12892 (F)
74%
3
2
3
NA12004 (F)
70%
4
NA12752 (M)
44%
2
3
NA12891 (M)
85%
5
11
NA10854 (F)
80%
2
4
NA10859 (F)
70%

Figure 3.4 *P4HA2* promoter/exon 1 DNA methylation patterns in LCLs. **A.** Location of the interrogated CGs with respect to *P4HA24* promoter, exon 1 and CG island. All features are shown in the context of the UCSC genome browser, assembly GRCh37/hg19 (http://genome.ucsc.edu). **B.** DNA methylation patterns of six LCLs. The LCL id, average percent methylation and average percent methylation for CG3 (in parentheses) are shown on the left. For other symbol descriptions please see figure 3.2.

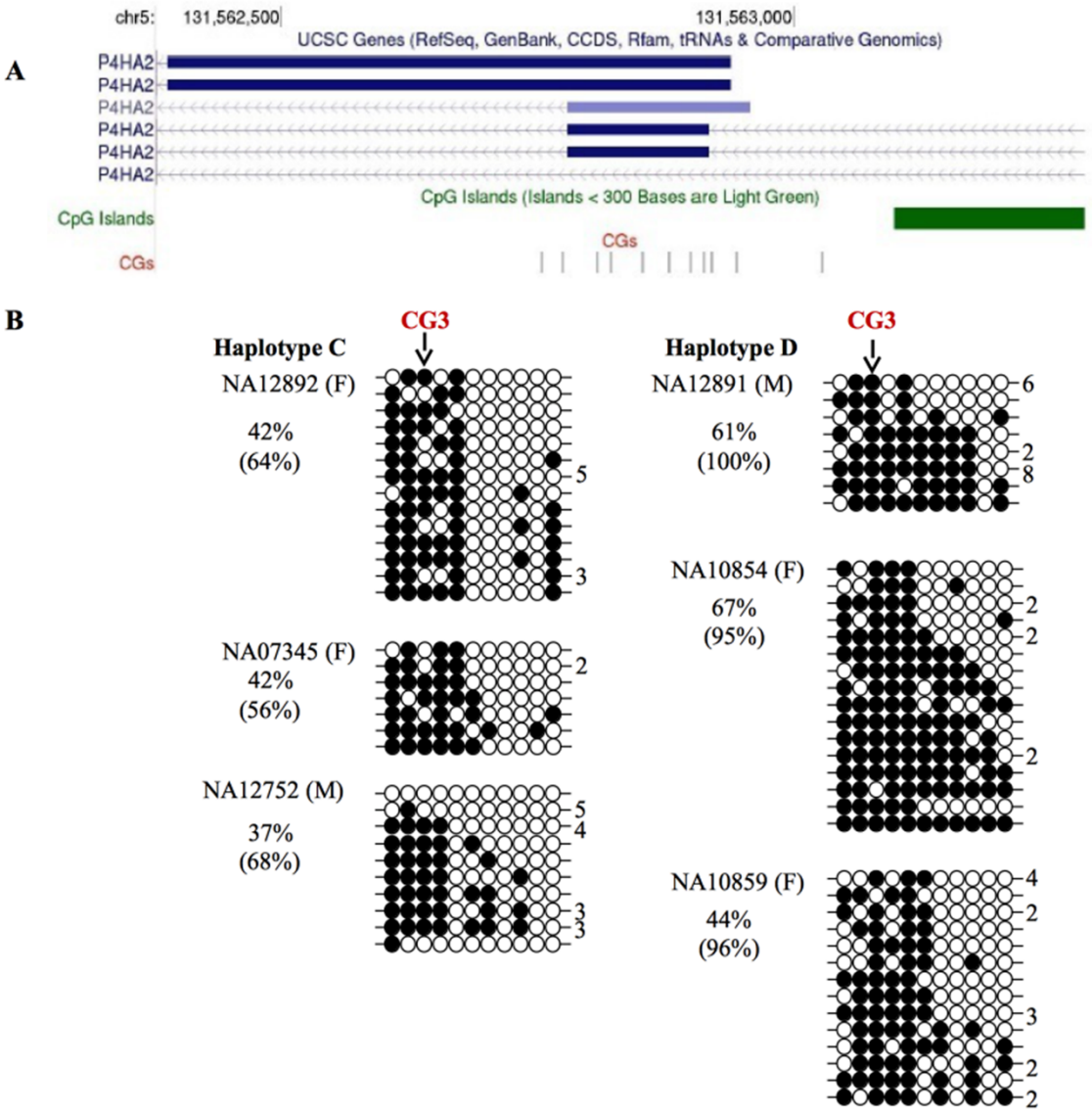

A
chr5: 131,562,500 131,563,000
UCSC Genes (RefSeq, GenBank, CCDS, Rfam, tRNAs & Comparative Genomics)
P4HA2
P4HA2
P4HA2
P4HA2
P4HA2
P4HA2
CpG Islands (Islands < 300 Bases are Light Green)
CpG Islands
CGs
CGs
B
Haplotype C
CG3
NA12892 (F)
42%
(64%)
5
3
NA07345 (F)
42%
(56%)
2
NA12752 (M)
37%
(68%)
5
4
3
3
Haplotype D
CG3
NA12891 (M)
61%
(100%)
6
2
8
NA10854 (F)
67%
(95%)
2
2
2
NA10859 (F)
44%
(96%)
4
2
3
2
2

Figure 3.5 *SLC22A5* promoter/exon 1 DNA methylation patterns in LCLs. **A.** Location of the interrogated CGs by bisulfite sequencing and pyrosequencing methylation assays is shown with respect to *SLC22A5* promoter, exon 1 and CG island. The black bar shows 7 differentially methylated CGs (CG31-CG37) that were analyzed using the pyrosequencing assay. All features are shown in the context of the UCSC genome browser, assembly GRCh37/hg19 (http://genome.ucsc.edu). **B.** DNA methylation patterns of eight LCLs. The LCL ids are shown on the left. For other symbol descriptions please see figure 3.2.

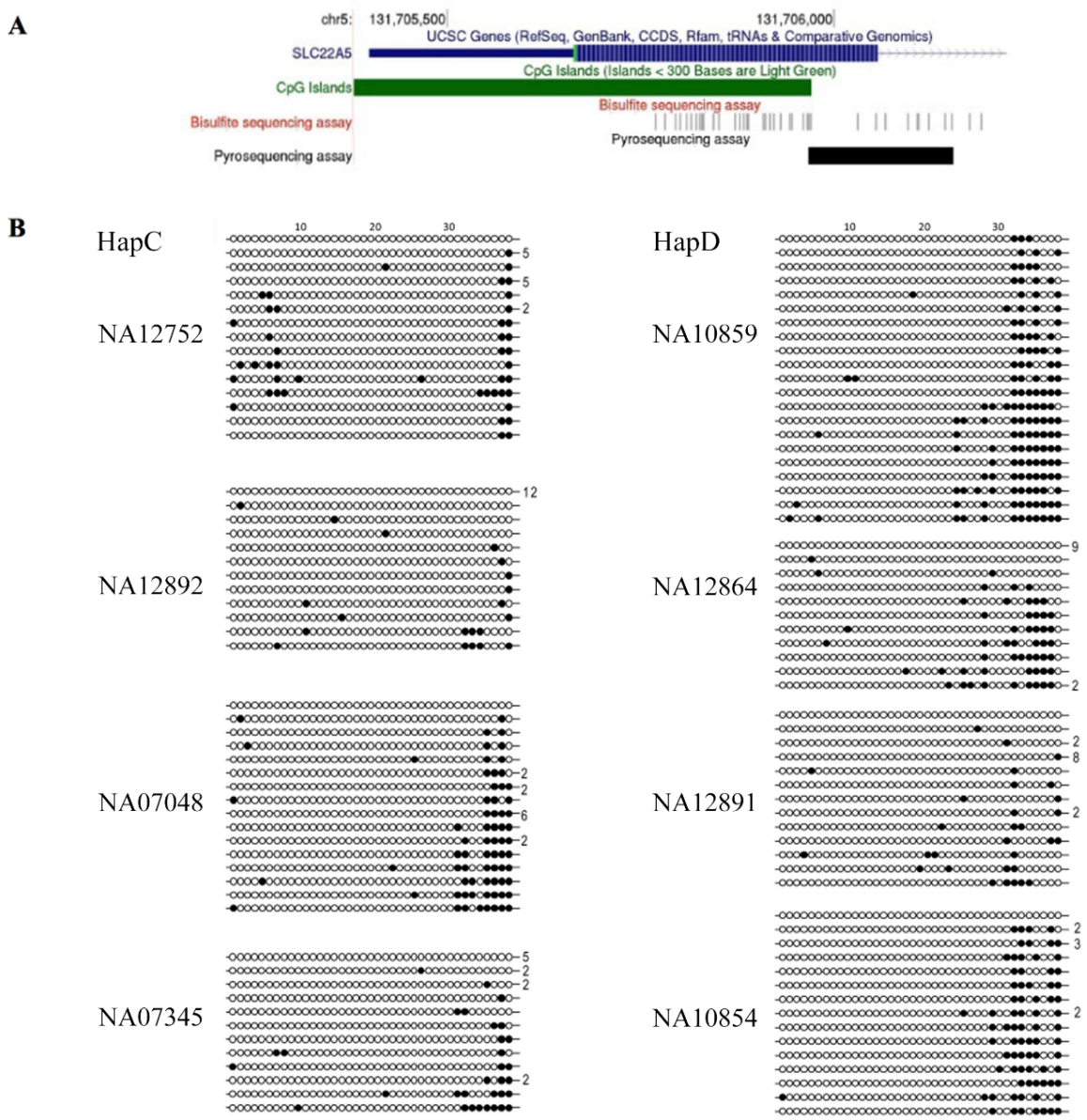

A
chr5: 131,705,500 131,706,000
UCSC Genes (RefSeq, GenBank, CCDS, Rfam, tRNAs & Comparative Genomics)
SLC22A5
CpG Islands (Islands < 300 Bases are Light Green)
CpG Islands
Bisulfite sequencing assay
Bisulfite sequencing assay
Pyrosequencing assay
Pyrosequencing assay
B
HapC
HapD
NA12752
NA10859
NA12892
NA12864
NA07048
NA12891
NA07345
NA10854

Figure 3.6 Optimization of concentration of 5-aza-dC treatment in HEK293T/17 and NuLi-1. **A.** Treatment HEK293T/17 cell line with 5-aza-dC. The bar graph shows the fold change in *H19* RNA levels compared to cells treated with 50% acetic acid without 5-aza- dC. The concentration of 50µM was selected for further experiments. **B.** Treatment NuLi-1 cell line with 5-aza-dC. The bar graph shows the fold change in *H19* RNA levels compared to cells treated with 0.5% DMSO alone. The concentration of 0.5µM was selected for further experiments.

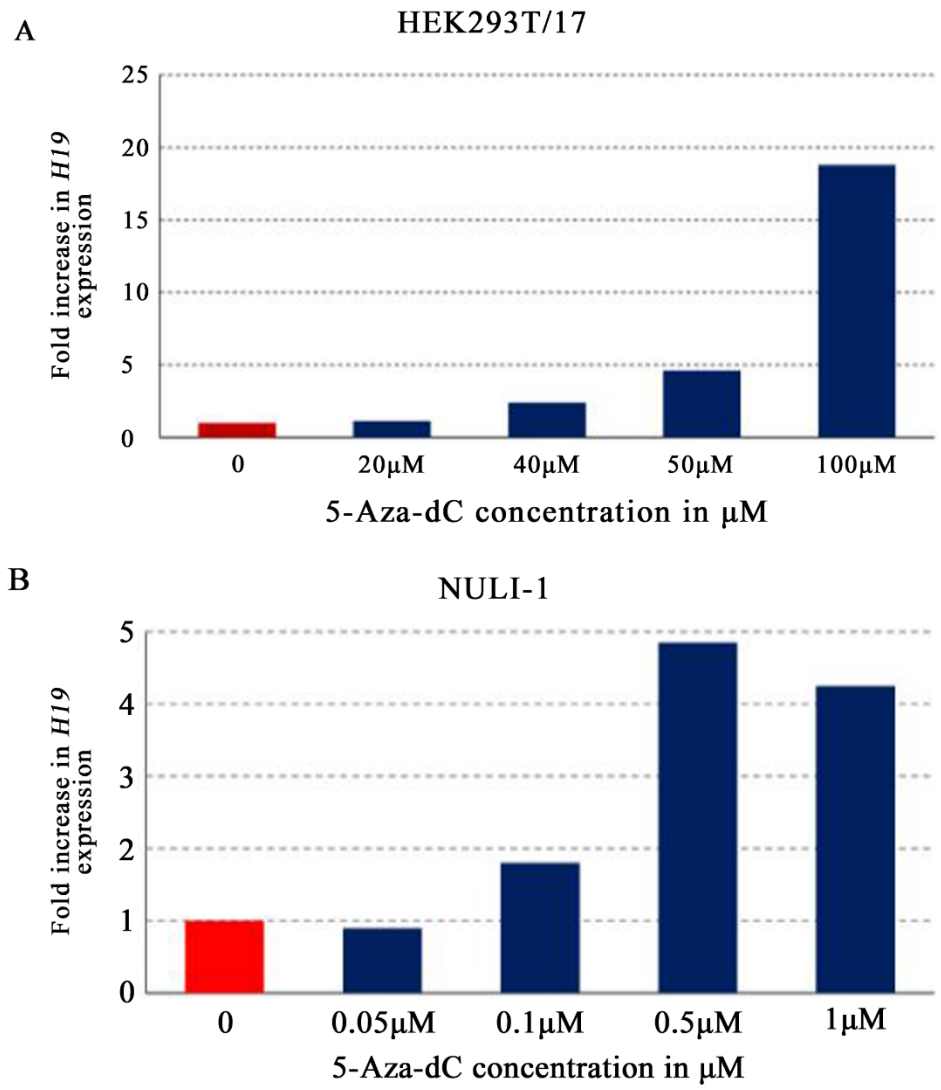


Figure 3.7 5-aza-dC treatment enhances promoter exon 1 *SLC22A5* gene expression but not the methylation level. **A.** Changes in expression levels of *H19* and *SLC22A5* genes after 5-aza-dC treatment in HEK293T/17 and NuLi-1. The y-axis shows fold change in 5-aza-dC treated cells compared to controls. Error bars show standard deviation. Asterisks indicate statistically significant change in expression in 5-aza-dC treated cells compared to controls (* $p < 0.05$). **B.** The bar graph shows the level of methylation patterns of the *SLC22A5* promoter exon1 region following treated HEK293T/17 with 5-aza-dC by using sodium bisulfite methylation analysis. No significant difference in *SLC22A5* DNA methylation level between 5-aza-dC treated cells and controls was observed.

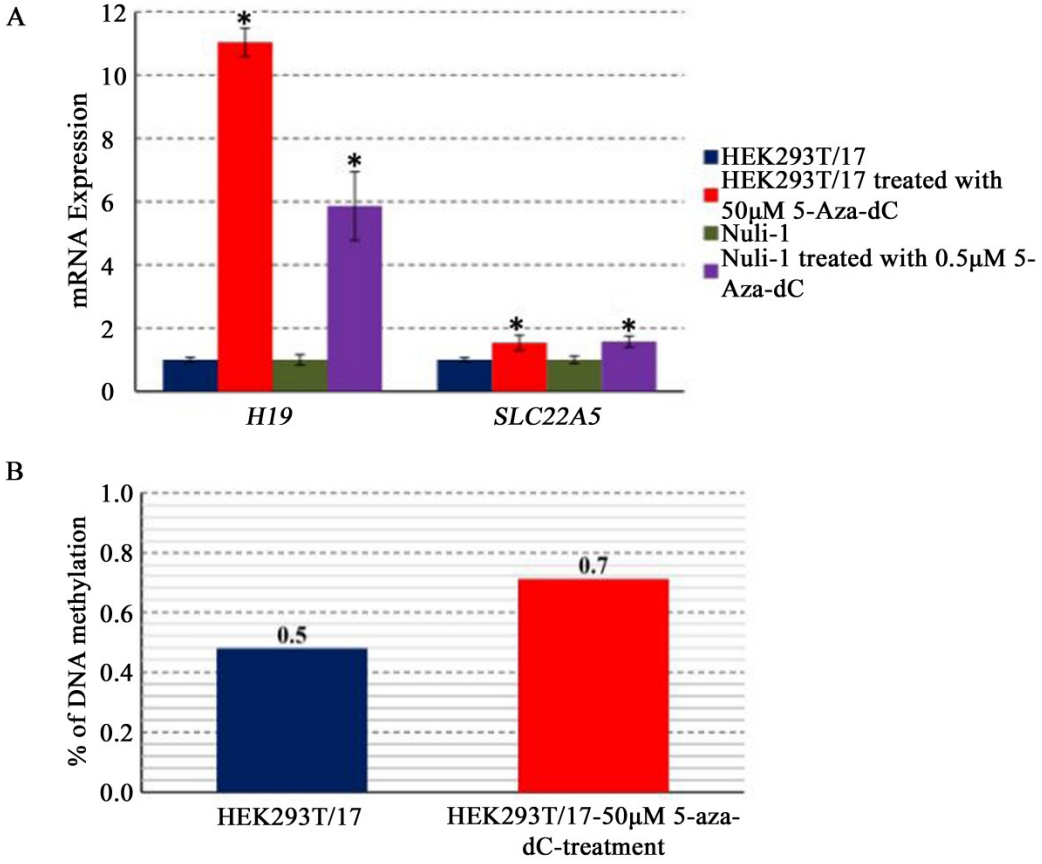
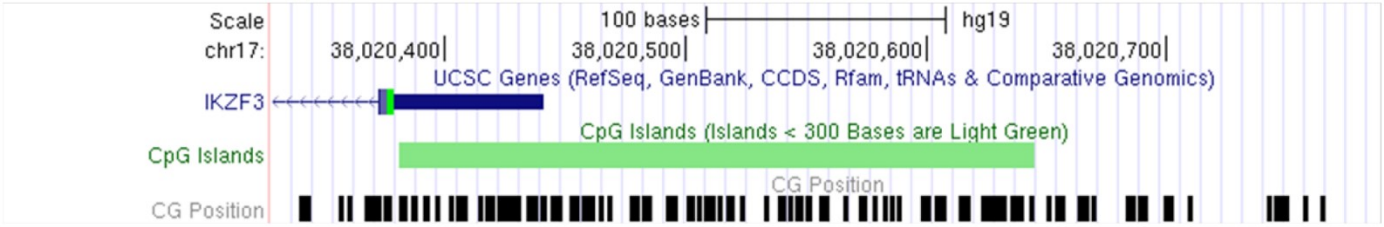


Figure 3.8 *IKZF3* promoter exon 1 methylation patterns in LCLs. **A.** Location of the interrogated CGs with respect to *IKZF3* promoter, exon 1 and CG island. All features are shown in the context of the UCSC genome browser, assembly GRCh37/hg19 (http://genome.ucsc.edu). **B.** DNA methylation profile of the *IKZF3* promoter does not depend upon the haplotype. Open circles represent unmethylated CG sites, filled black circles represent methylated CG sites and gray circles represent non-informative CGs. Numbers above the circles indicate the CG ID number. Each row represents an individual clone. The number shown on the right reflects the number of clones with same methylation pattern. The DNA sample ID is shown on the top.

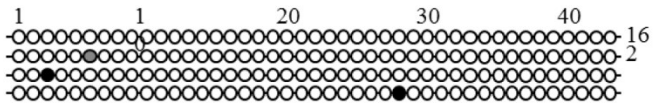
A



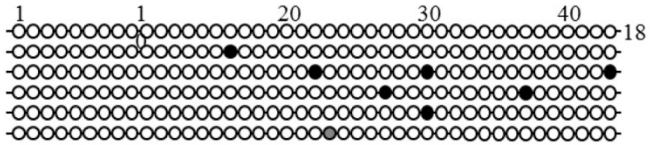
B

HapA

NA11995

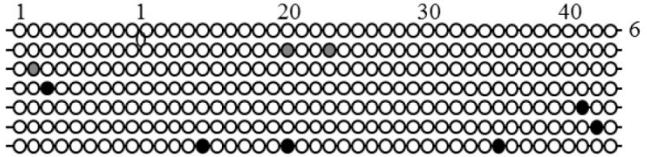


NA12761

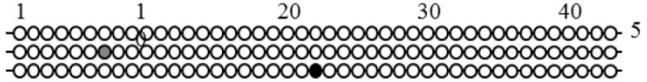


HapB

NA12239



NA12892



NA12146

Figure 3.9 *GSDMA* promoter/exon 1 DNA methylation patterns in LCLs. **A.** Location of the interrogated CGs by bisulfite sequencing and pyrosequencing methylation assays is shown with respect to *GSDMA* promoter, exon1. The black bar shows 3 differentially methylated CGs (CG1-CG3) that were analyzed using the pyrosequencing assay. All features are shown in the context of the UCSC genome browser, assembly GRCh37/hg19 (http://genome.ucsc.edu). **B.** Results of methylation analysis of the *GSDMA* promoter, exon and part of intron 1. Each panel corresponds to results from a single individual. Each circle represents a single CG pair; filled circles represent methylated CGs, open circles represent unmethylated CGs; each row represents a clone and therefore corresponds to a single allele, the number of clones with same methylation profile is shown on the right. Percentages on the right show the average percent methylation across all CGs and all alleles.

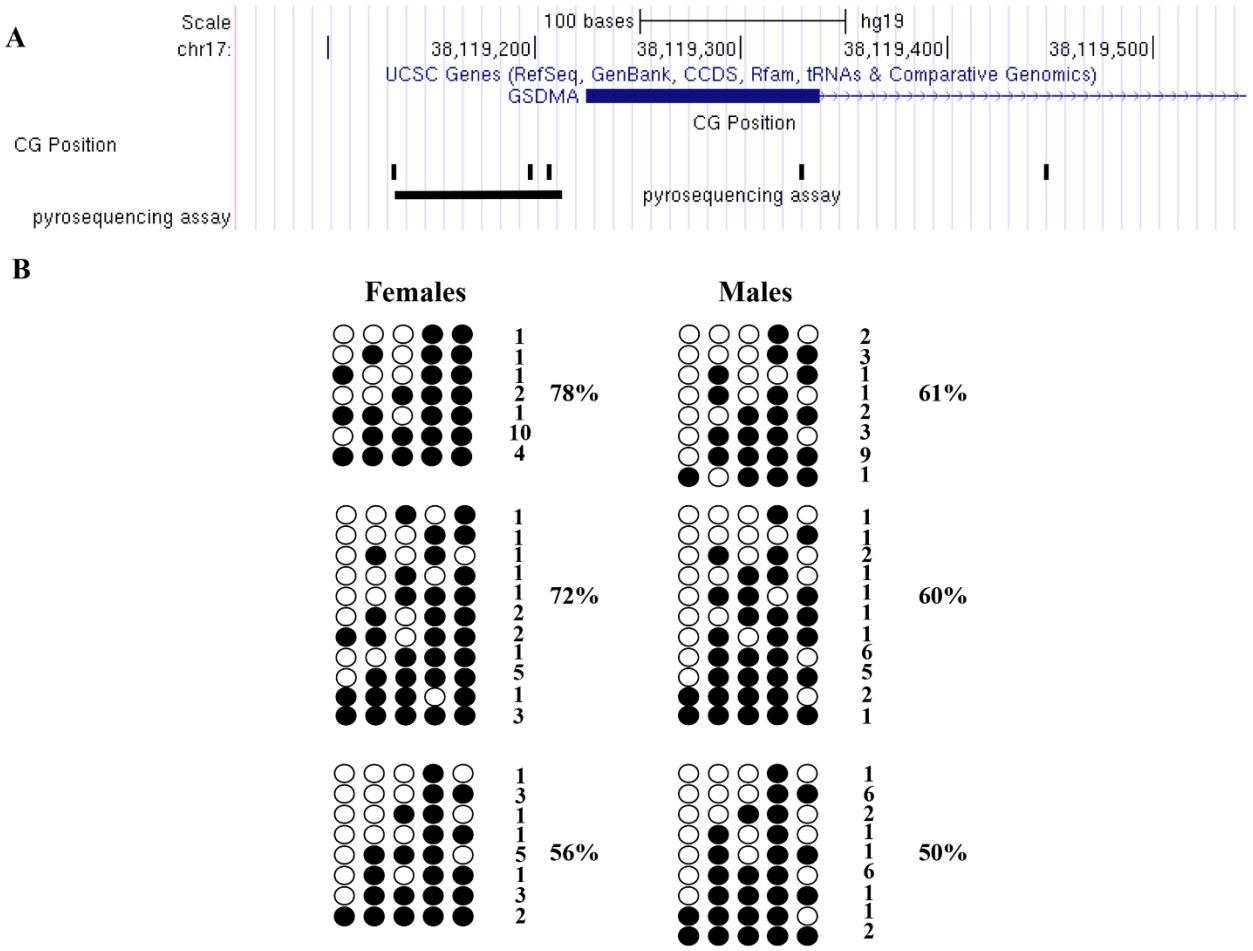

A
Scale
chr17:
100 bases
hg19
38,119,200
38,119,300
38,119,400
38,119,500
UCSC Genes (RefSeq, GenBank, CCDS, Rfam, tRNAs & Comparative Genomics)
GSDMA
CG Position
CG Position
pyrosequencing assay
pyrosequencing assay
B
Females
Males
1 1 1 2 1 10 4
78%
2 3 1 1 2 3 9 1
61%
1 1 1 1 1 2 2 1 5 1 3
72%
1 1 2 1 1 1 6 5 2 1
60%
1 3 1 1 5 1 3 2
56%
1 6 2 1 1 6 1 1 2
50%

Figure 3.10 *CSF3* promoter region DNA methylation patterns in the peripheral blood cells of three females and three males. **A.** Positions of CGs, primers and SNP within the context of the UCSC browser, assembly GRCh37/hg19 (http://genome.ucsc.edu). **B.** Results of methylation analysis of the *CSF3* promoter, exon and intron 1. Each panel corresponds to results from a single individual. Each circle represents a single CG pair; filled circles represent methylated CGs, open circles represent unmethylated CGs; each row represents a clone and therefore corresponds to a single allele, the number of clones with same methylation profile is shown on the right. Percentages on the right show the average percent methylation across all CGs and all alleles.

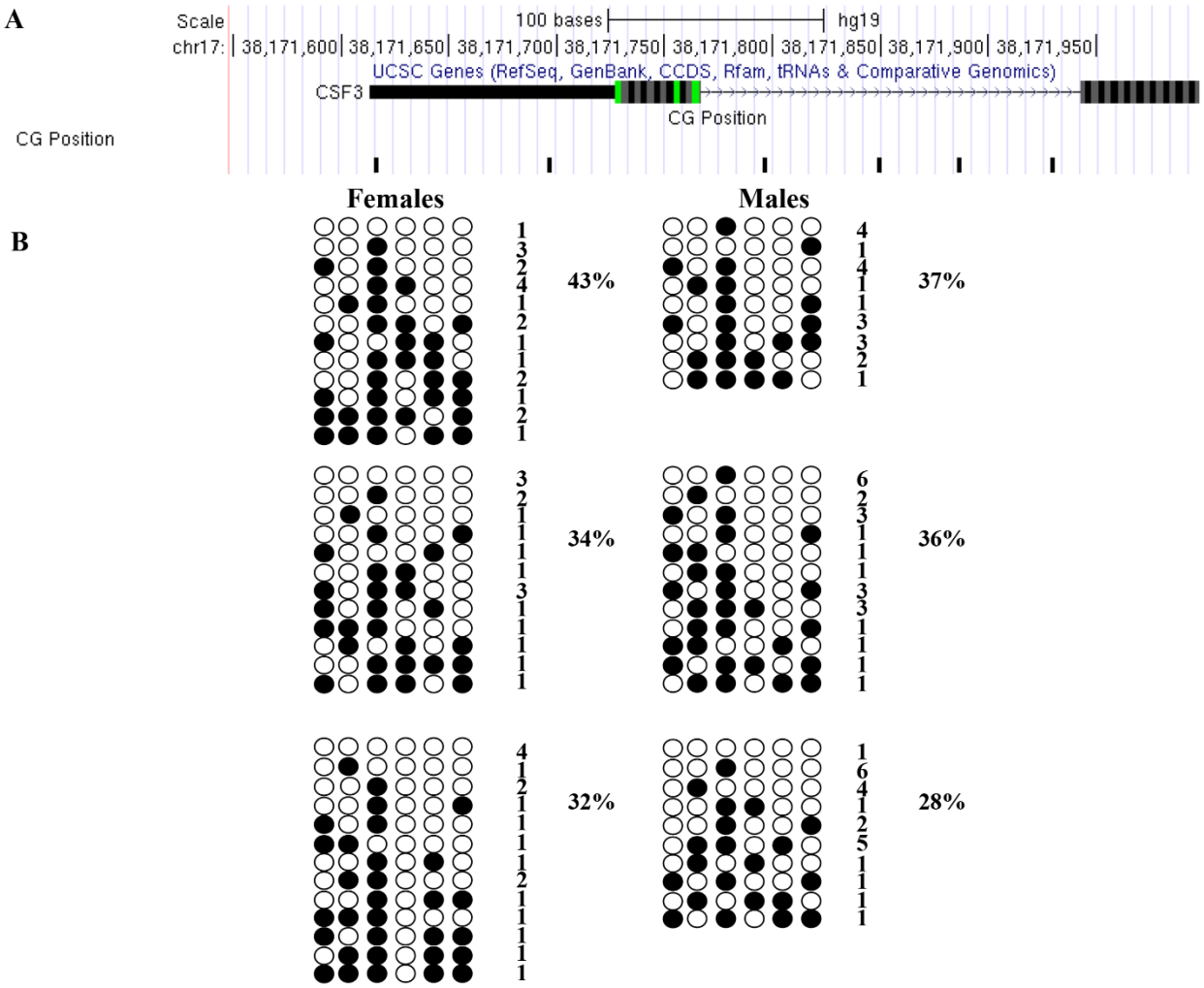
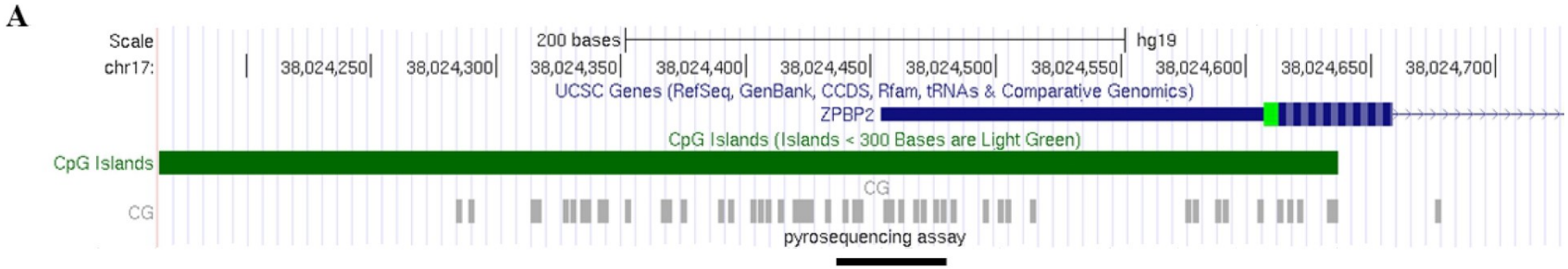

A
Scale
100 bases
hg19
chr17:
38,171,600
38,171,650
38,171,700
38,171,750
38,171,800
38,171,850
38,171,900
38,171,950
UCSC Genes (RefSeq, GenBank, CCDS, Rfam, tRNAs & Comparative Genomics)
CSF3
CG Position
CG Position
B
Females
Males
43%
37%
34%
36%
32%
28%

Figure 3.11 *ZPBP2* promoter/exon 1 DNA methylation patterns in LCLs. **A.** Location of the interrogated CGs by bisulfite sequencing and pyrosequencing methylation assays is shown with respect to *ZPBP2* promoter, exon1. The black bar shows 11 differentially methylated CGs that were analyzed using the pyrosequencing assay. All features are shown in the context of the UCSC genome browser, assembly GRCh37/hg19 (http://genome.ucsc.edu). **B.** Variable DNA methylation of *ZPBP2* promoter. Filled circles represent methylated cytosines, open circles represent unmethylated cytosines in CG pairs. Each row represents the methylation pattern of a single clone. Percentages on the right CGs group show the average percent methylation across all CGs and all alleles.


A
Scale
chr17:
200 bases
hg19
38,024,250
38,024,300
38,024,350
38,024,400
38,024,450
38,024,500
38,024,550
38,024,600
38,024,650
38,024,700
UCSC Genes (RefSeq, GenBank, CCDS, Rfam, tRNAs & Comparative Genomics)
ZPBP2
CpG Islands (Islands < 300 Bases are Light Green)
CpG Islands
CG
CG
pyrosequencing assay

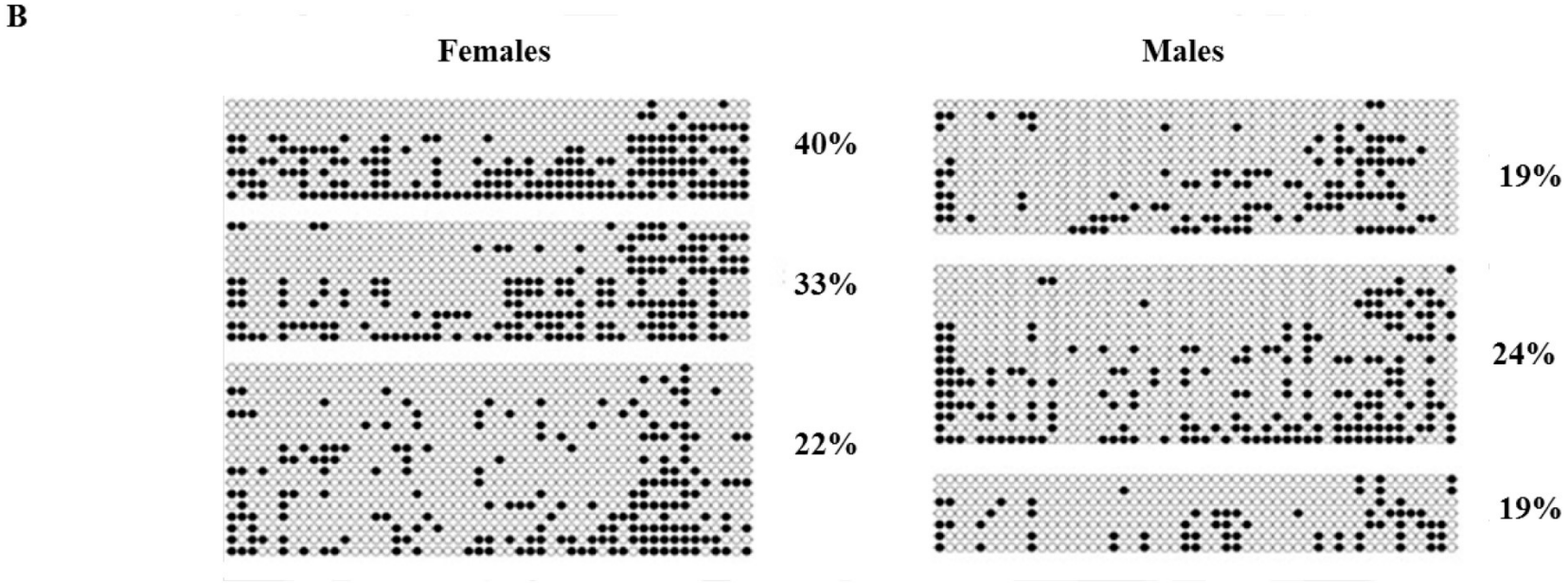

B
Females
Males
40%
33%
22%
19%
24%
19%

Figure 3.12 Effect of sex on average DNA methylation levels in *ZPBP2, GSDMA* and *CSF3*. Red bars show average methylation levels in females, blue bars show average methylation levels in males. Error bars show standard deviation. For *ZPBP2*, methylation patterns were determined in ten males and nine females, males had significantly lower average methylation levels of *ZPBP2* promoter region compared to females (t-test statistics, *P* = 1.0E-4); For *GSDMA*, methylation patterns were determined in three males and six females; for CSF3 in seven males and three females. DNA methylation of *GSDMA* and *CSF3* promoters varied among individuals, but did not show statistically significant sex-specific differences when the number of samples was increased (t test statistics, p = 9.9E-2 for *GSDMA* and p = 2.8E-1 for *CSF3*).

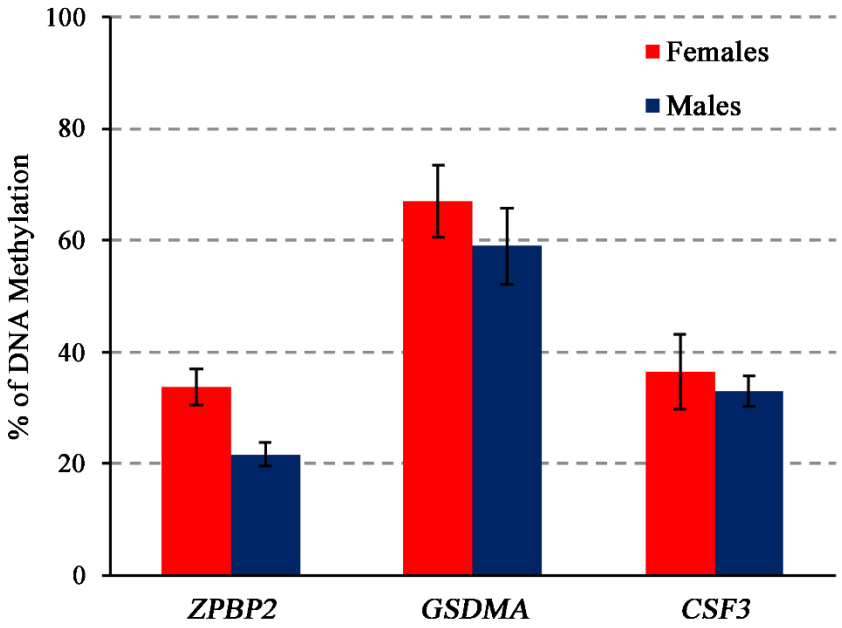


Figure 3.13 5-aza-dC treatment enhances genes expression**. A.** 5-aza-dC treated NuLi-1 cells show reduced cell growth and apoptosis four days after treatment. Arrowheads point to dead cells. The images were captured using Leica DMI6000 B inverted microscope **B.** Changes in expression levels of 17q21 genes after 5-aza-dC treatment. The y-axis shows fold change in 5-aza-dC treated cells compared to controls. Error bars show standard deviation. Asterisks indicate statistically significant change in expression in 5-aza-dC treated cells compared to controls (* $p < 0.05$). **C.** Allelic expression in 17q21 genes after 5-aza-dC treatment. Arrows show positions of transcribed SNPs in those genes where allelic expression changed post 5-aza-dC treatment. In *ZPBP2*, 5-aza-dC treatment causes reactivation of the HapA allele. In *ORMDL3* it causes a switch in allelic preference (Moussette et al., 2017).

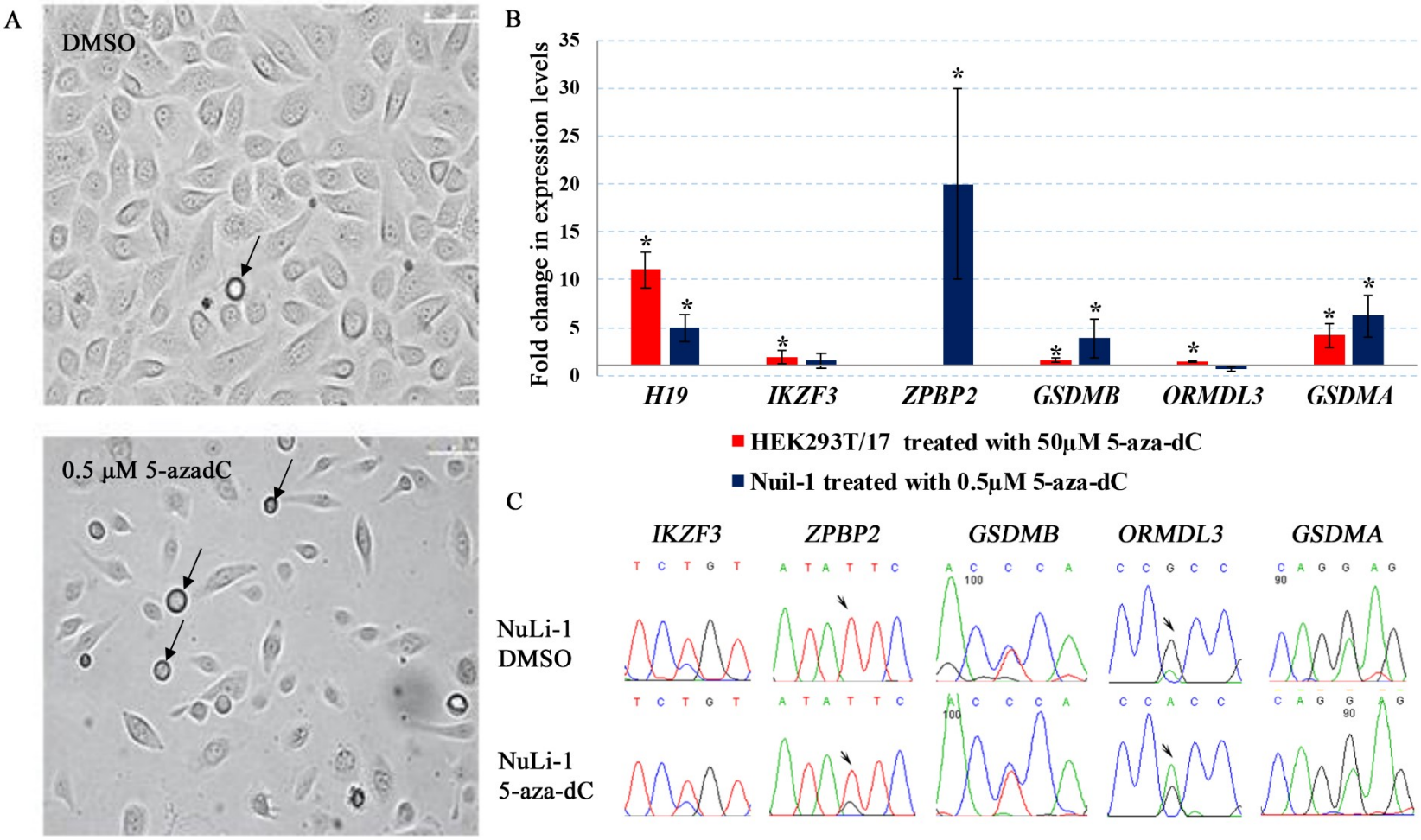

A
DMSO
0.5 µM 5-azadC
B
Fold change in expression levels
35
30
25
20
15
10
5
0
H19
IKZF3
ZPBP2
GSDMB
ORMDL3
GSDMA
HEK293T/17 treated with 50µM 5-aza-dC
Nuil-1 treated with 0.5µM 5-aza-dC
C
IKZF3
ZPBP2
GSDMB
ORMDL3
GSDMA
NuLi-1
DMSO
NuLi-1
5-aza-dC

Figure 3.14 DNA methylation profiles of genes promoter region in control (DMSO) and 5-aza-dC treated cells. **A.** DNA methylation patterns of the *ZPBP2* promoter in NuLi-1 cells. Filled circles represent methylated CGs, open circles represent unmethylated CGs. Each row represents a clone. Data are divided by allele; allelic percent methylation is shown below the diagram. Type of treatment is shown on top. Arrow showed the position of CG31 (Corresponding to CG6 in pyrosequencing assays (table A.1)) that showed the highest allelic differences in methylation. **B.** *GSDMA* promoter methylation changes after 5-aza-dC treatment. Filled circles represent methylated CGs, open circles represent unmethylated CGs. Each row represents a clone, the number on the right indicates the number of clones with a particular methylation pattern. Data are divided by allele; allelic percent methylation is shown below the diagram. Type of treatment and average DNA methylation are shown on top. **C.** DNA methylation patterns of the *ORMDL3* promoter in NuLi-1 cells. Filled circles represent methylated CGs, open circles represent unmethylated CGs. Each row represents a clone, the number on the right indicates the number of clones with a particular methylation pattern (Moussette et al., 2017).

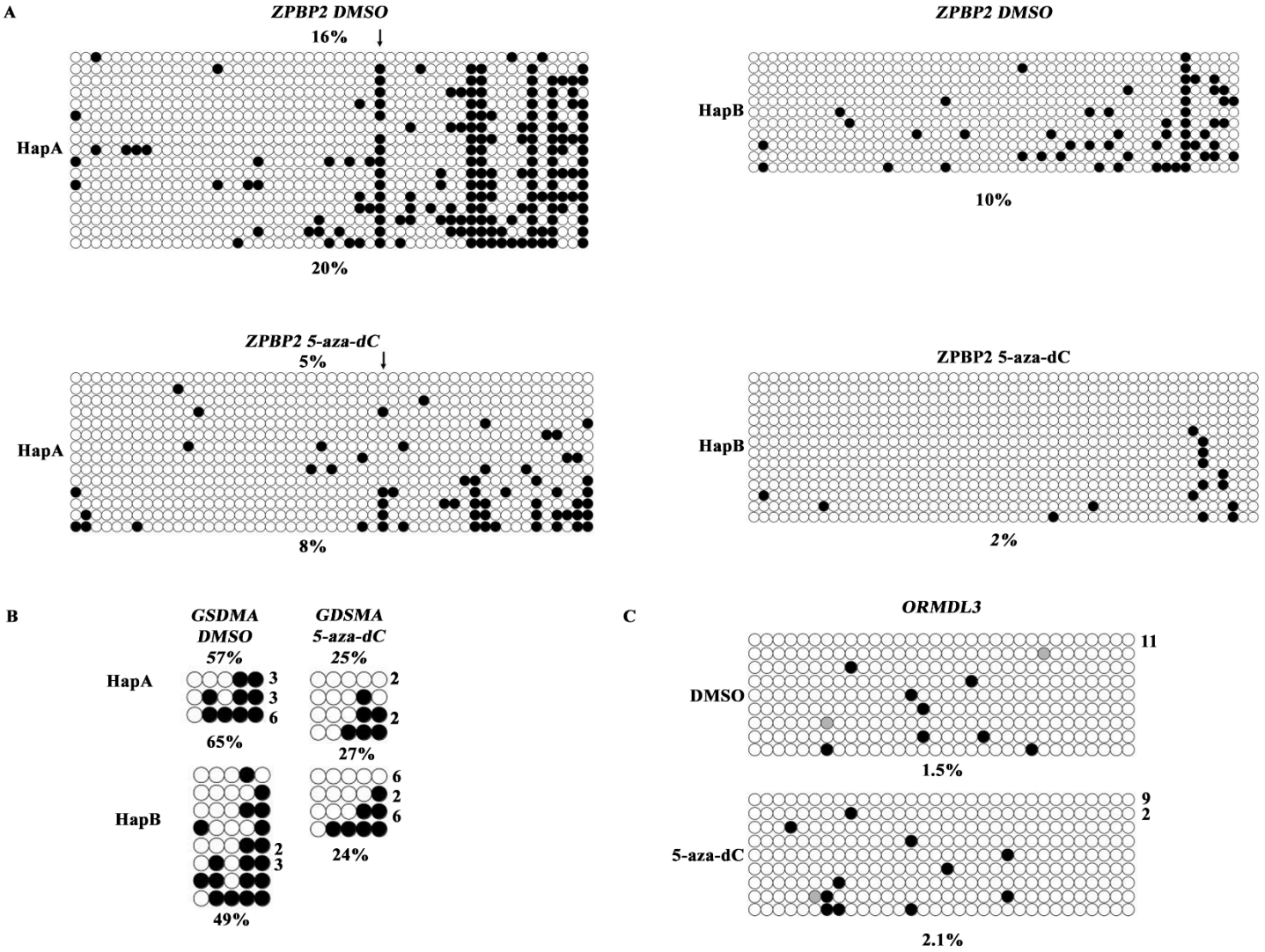

A
ZPBP2 DMSO
16%
HapA
20%
ZPBP2 DMSO
HapB
10%
ZPBP2 5-aza-dC
5%
HapA
8%
ZPBP2 5-aza-dC
HapB
2%
B
GSDMA
DMSO
57%
HapA
3
3
6
65%
HapB
2
3
49%
GDSMA
5-aza-dC
25%
2
2
27%
6
2
6
24%
C
ORMDL3
DMSO
11
1.5%
5-aza-dC
9
2
2.1%

Figure 3.15 *ZPBP2* promoter methylation levels in 21 fibroblast cell lines were analyzed by bisulfite sequencing. The cell lines included different combinations of sex chromosome dosage and sex phenotype: 45, X female, 46, XY males and females, 46, XX males and females, and 48, XXY males. **A**. No significant change detected in methylation level in *ZPBP2* region in respect to sex phenotype. **B**. No significant change detected in methylation level in *ZPBP2* region in respect to absence or presence *SRY* gene. **C**. DNA methylation of *ZPBP2* is significantly increased with the number of X chromosomes. P values in (A, B and C) were calculated by Student's t-test. **D**. Increase number of X chromosome in the absence of *SRY* gene is associated with *ZPBP2* higher methylation levels. All fibroblasts were genotyped for *SRY*. Each circle represents the methylation level of one individual fibroblasts. *P* value was calculated by one way ANOVA.

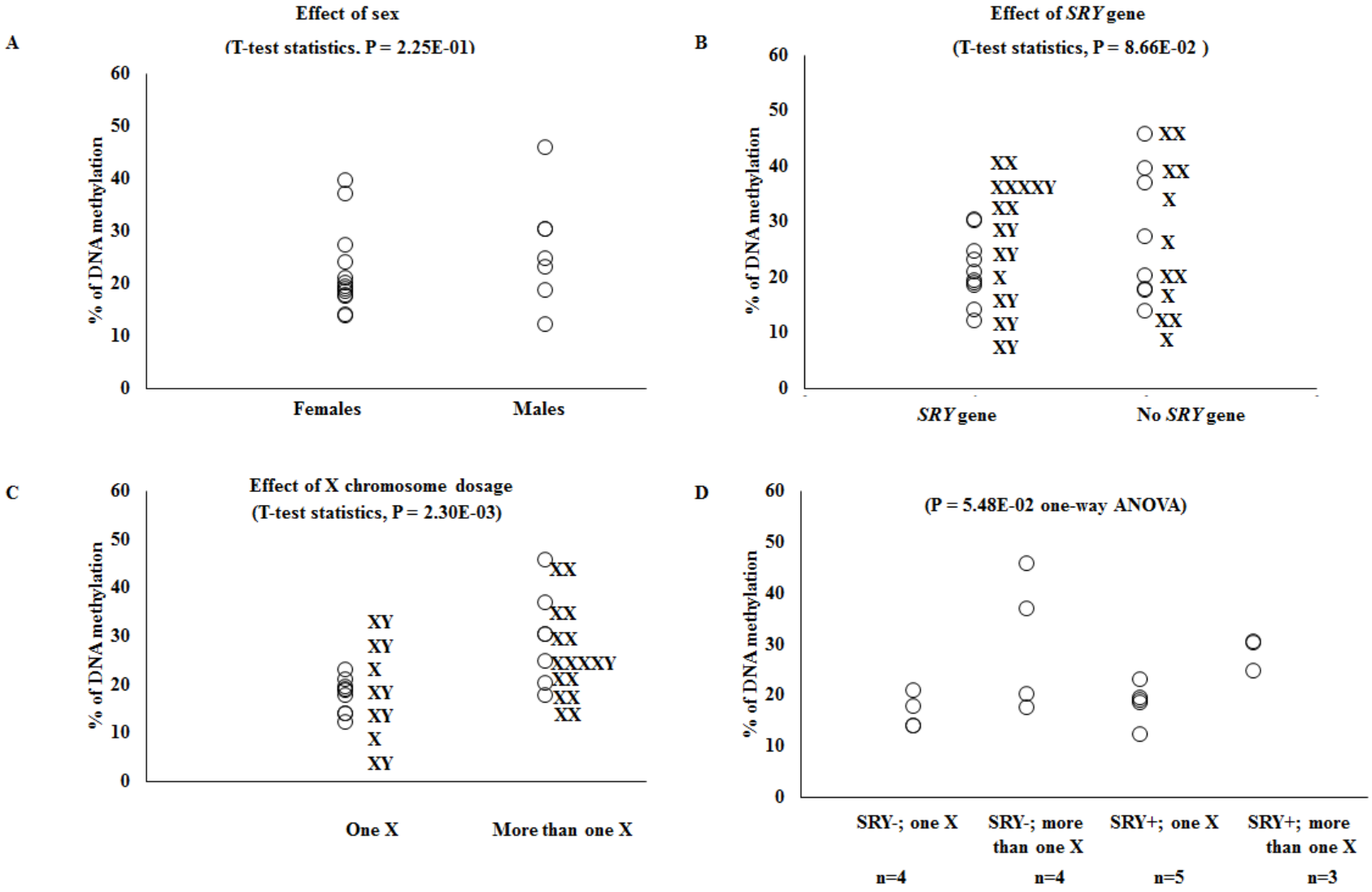

A
Effect of sex
(T-test statistics. P = 2.25E-01)
% of DNA methylation
0
10
20
30
40
50
60
Females
Males
B
Effect of SRY gene
(T-test statistics, P = 8.66E-02)
% of DNA methylation
XX
XXXXY
XX
XY
XY
X
XY
XY
XY
XX
XX
X
X
XX
X
XX
X
SRY gene
No SRY gene
C
Effect of X chromosome dosage
(T-test statistics, P = 2.30E-03)
% of DNA methylation
XY
XY
X
XY
XY
X
XY
XX
XX
XX
XXXXY
XX
XX
XX
One X
More than one X
D
(P = 5.48E-02 one-way ANOVA)
% of DNA methylation
SRY-; one X
SRY-; more than one X
SRY+; one X
SRY+; more than one X
n=4
n=4
n=5
n=3

Figure 3.16 *MECP2* mutations are associated with lower methylation levels in the *ZPBP2* promoter region. The bar chart represents the percent of *ZPBP2* DNA methylation level. The red bar shows individuals with more than one X chromosome. The blue bar shows individuals with one X chromosome. Error bars show standard deviation. There is a statistically significant difference in *ZPBP2* methylation level between samples with one X chromosome and more than one X chromosome. There is lost in *ZPBP2* differential methylation in females with Rett syndrome.

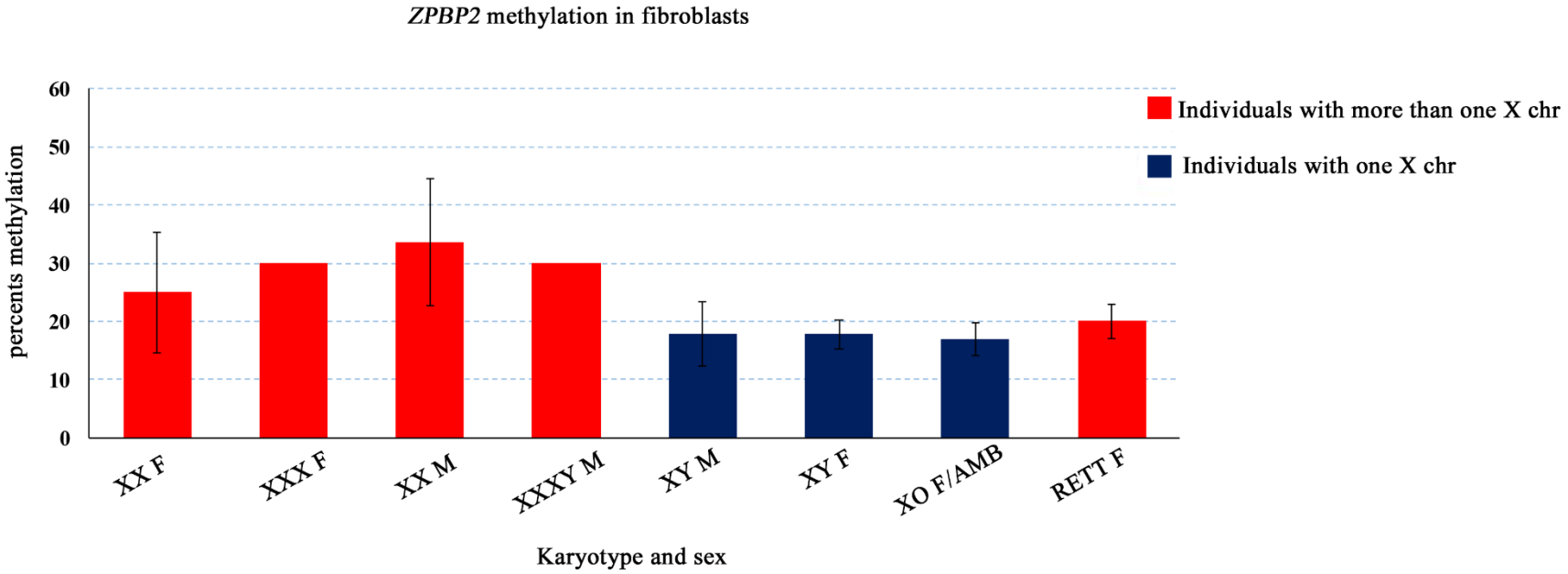
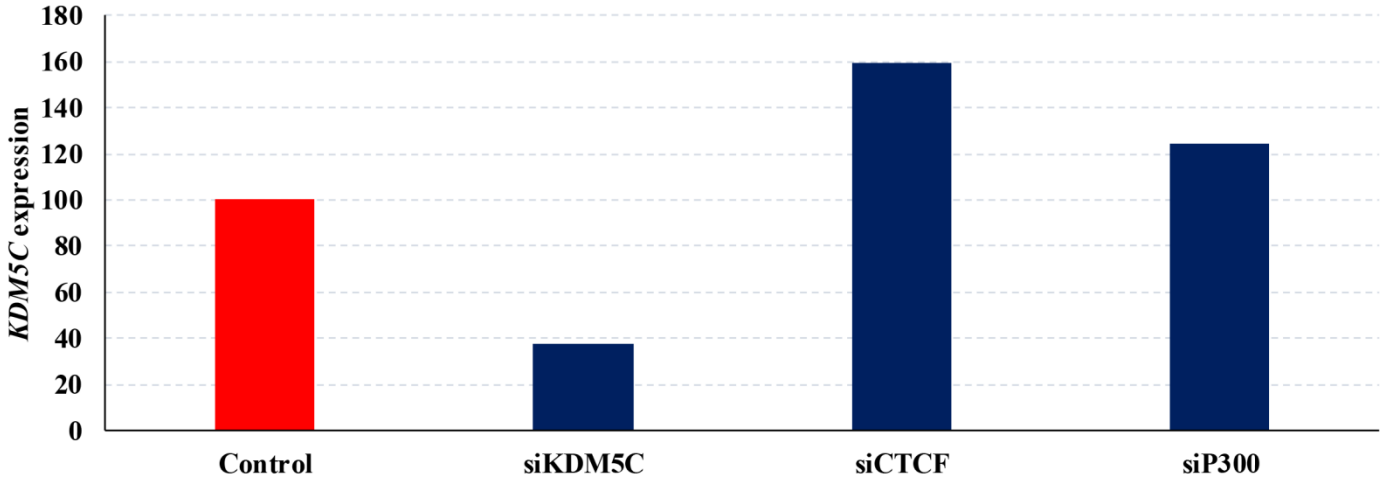

ZPBP2 methylation in fibroblasts
percents methylation
60
50
40
30
20
10
0
XX F
XXX F
XX M
XXXY M
XY M
XY F
XO F/AMB
RETT F
Karyotype and sex
Individuals with more than one X chr
Individuals with one X chr

Figure 3.17 Expression levels of genes following transfection of HEK293T/17 with siKDM5C. **A.** To confirm the specificity of siKDM5C construct, *KDM5C* expression was assessed in HEK293T/17 transfected with siKDM5C, siCTCF and siP300 in separate. The mRNA level of *KDM5C* was measured by quantitative RT-PCR and normalized to the GAPDH RNA levels (n=2). *KDM5C* showed reduce in expression 2.7-fold only after HEK293T/17 transfected with siKDM5C. **B.** Expression levels of *KDM5C, DNMT1*, *DNMT3A, IKZF3, GSDMB, ORMDL3 CACYBP, FBXL5* and *SCMH1.* Expression levels were measured following KDM5C-siRNA interference in HEK293T/17 cell by quantitative RT-PCR and normalized to the 18S RNA levels in three replicate experiments. The mRNA level of *KDM5C* showed 4-fold decrease in expression after transfection. *IKZF3* showed 2.5-fold increase in expression after transfection. There was no change in expression levels in *DNMT3A, DNMT1*, *GSDMB, ORMDL3 CACYBP, FBXL5* and *SCMH1* between control and transfected HEK293T/17. Asterisks indicate statistically significant change in expression in transfected HEK293T/17 cells compared to controls (* $p < 0.05$).

A


180
160
140
120
100
80
60
40
20
0
KDM5C expression
Control
siKDM5C
siCTCF
siP300

B

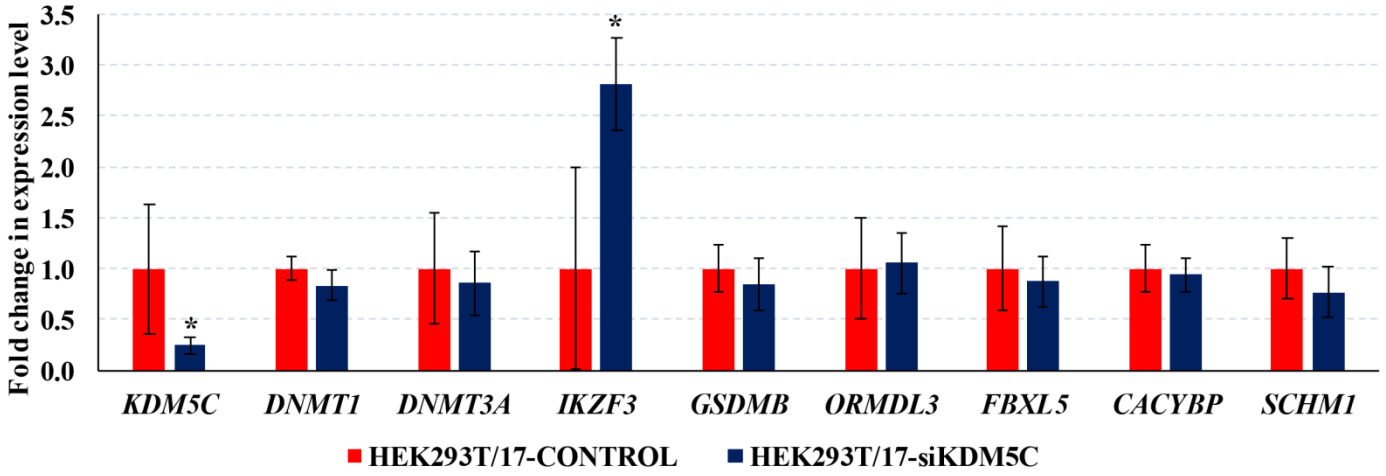
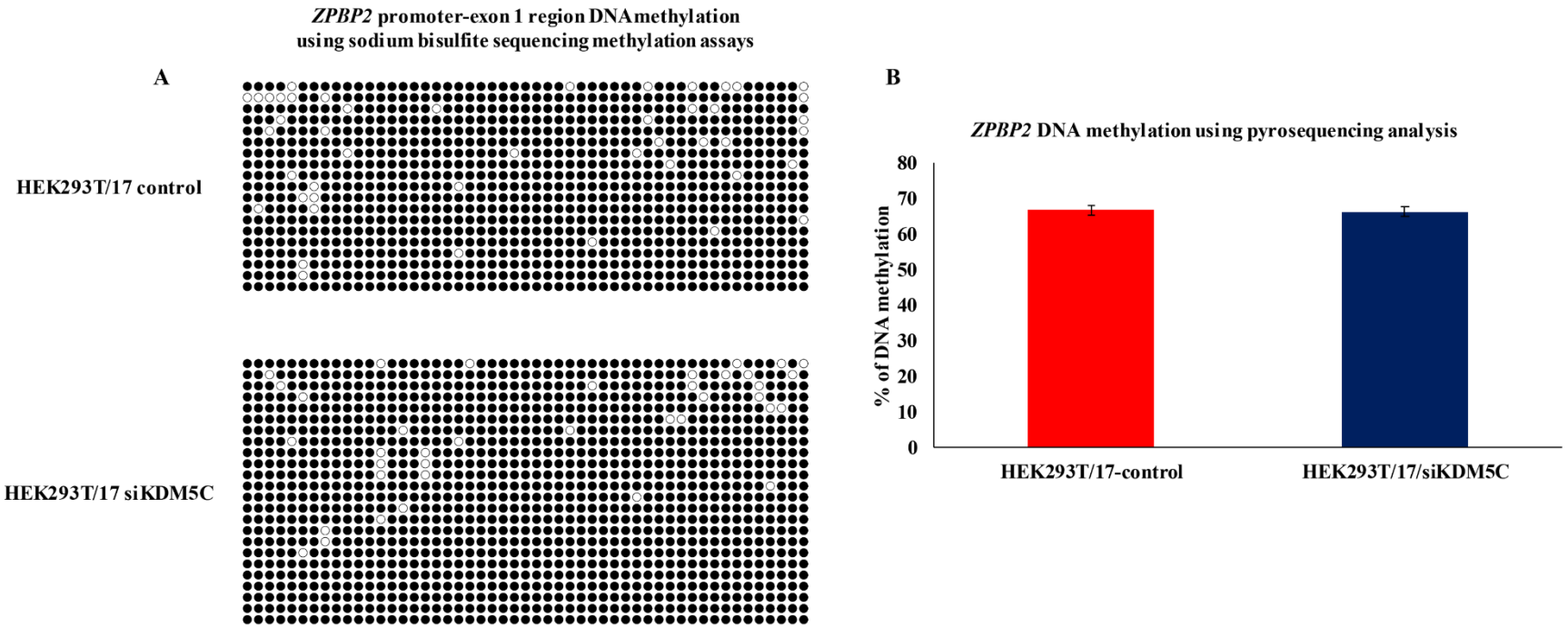

3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Fold change in expression level
*
*
KDM5C
DNMT1
DNMT3A
IKZF3
GSDMB
ORMDL3
FBXL5
CACYBP
SCHM1
HEK293T/17-CONTROL
HEK293T/17-siKDM5C

Figure 3.18 *ZPBP2* DNA methylation level of HEK293T/17 cell line after transfection with siKDM5C. **A.** Bead diagrams show methylation patterns of the *ZPBP2* promoter by using sodium bisulfite methylation analysis: each circle represents a single CG pair. Open circles represent unmethylated, filled methylated CGs. Each row represents one clone. The top panel represents the HEK293T/17 control and the bottom panel the *KDM5C* knock-down (siKDM5C). *ZPBP2* promoter is highly methylated in HEK293T/17. No significant difference in DNA methylation between siKDM5-knock down and control was observed. **B.** Red bars show average methylation levels in HEK293T/17 control, blue bars show average methylation levels in transfected HEK293T/17 with siKDM5C by using pyrosequencing methylation assay. Error bars show standard deviation. No significant difference in average methylation levels of *ZPBP2* promoter region in siKDM5knock down compared to control (t test statistics, p = 7.18E-01). The result shown is representative of three similar experiments.


ZPBP2 promoter-exon 1 region DNA methylation
using sodium bisulfite sequencing methylation assays
A
HEK293T/17 control
HEK293T/17 siKDM5C
B
ZPBP2 DNA methylation using pyrosequencing analysis
% of DNA methylation
80
70
60
50
40
30
20
10
0
HEK293T/17-control
HEK293T/17/siKDM5C

CHAPTER 4: DISCUSSION

In this study, promoter methylation of genes that previously showed allelic bias in expression were analyzed at two genomic regions, 5q31 and 17q21, which are associated with asthma. In both regions, the association with asthma was influenced by sex. Hence, we suggested that these regions contain genotype (gDMR) and/or sex influences on DNA methylation levels located in genes that have differences in allelic expression.

4.1. Local genotype influences DNA methylation at asthma-associated regions in *SLC22A5* at 5q31 region

DNA methylation of the promoter/exon 1 of genes with allelic bias in expression at the 5q31 region that is associated with asthma was analyzed. We found one gDMR in the *SLC22A5* promoter region that shows a lower methylation level on the same asthma-associated haplotype HapC in the whole blood samples, which was previously reported to be associated with higher allelic expression in LCLs (Ge et al., 2009). This supports the notion that DNA methylation functions as an epigenetic factor regulating gene expression (Holliday and Pugh, 1975, Jones and Taylor, 1980). *SLC22A5* is located in the cytokine cluster region, expressed in many different tissues, including lung, and found at high levels in the apical side of airway epithelial cells in tracheal tissue and in alveolar epithelial cells (Nakamura et al., 2010). It plays an essential role in the elimination of several environmental toxins while also facilitating the trans-membrane transport of organic cations and carnitine, which is involved in beta-oxidation of long-chain fatty acids in mitochondria (reviewed in (Mongioi et al., 2016)). *SLC22A5* is not only essential for carnitine transportation, but it is also involved in the recognition of clinically important medications (Grigat et al., 2009, Jong et al., 2011). Studies have suggested that *SLC22A5* is also contributed in the delivery of cationic bronchodilator drugs to the airway tissue used for treatment of pulmonary diseases, such as asthma (Horvath et al., 2007) (Nakamura et al., 2010). However, mechanisms regulating *SLC22A5* expression are not fully understood. Since *SLC22A5* is highly enriched in the apical side of the airway epithelium, which is considered a barrier through which inhaled cationic bronchodilator medications must pass to reach targeted receptors, individuals carrying HapC may have a good response to medication due to high *SLC22A5* expression. In addition, understanding the mechanism of *SLC22A5* regulation should provide information that can be used to improve the effectiveness of inhaled drugs for the treatment of asthma.

It has been reported in several studies that genetic variation between populations contributes to inter-individual variation in DNA methylation levels in different cell types (Bell et al., 2011) (Dayeh et al., 2013) (Zhang et al., 2010) (Gibbs et al., 2010). A complete understanding of how genetic polymorphisms influence DNA methylation levels in *cis* is important, as it could help explain plausible reasons behind the allelic bias of gene transcription and potential targets for therapy (van Eijk et al., 2012) (Zhang et al., 2009). However, the exact mechanism that leads to genotype-dependent changes in DNA methylation levels remains elusive. CpG-SNPs (single nucleotide polymorphisms that abolish or create a CpG site) influence epigenetic interactions in *cis*, probably by modulating regulatory elements (Shoemaker et al., 2010). DNA methylation level was regulated by genotype at the transient receptor potential cation channel, subfamily C, member 3 (*TRPC3*) alternative promoter. The rare C allele of the rs13121031 SNP located in CGI decreased methylation of surrounding CpG sites compared to the G allele in four different tissues, which negatively correlated with the C allele expression that was found at a higher frequency in idiopathic ataxia patients compared to controls (Martin-Trujillo et al., 2011). Another study showed that DNA methylation of a CpG-SNP created by the C allele rs144383 in the 5′UTR of the *GDF5* gene attenuated the repressive effects of SP1 and SP3 transcription factors, which caused an expression bias between both alleles (Reynard et al., 2014). In a study on 52 parent-child trios, the methylation levels of about 412,000 CpG sites in the human genome were characterized, they found that most heritable CG methylation levels could be associated to either CG-SNPs or *trans*-effects from regulatory elements (Plongthongkum et al., 2014). We hypothesized that a SNP that causes gain/loss of a CG site may be responsible for changes in methylation levels. The 2-kb region that flanks the gDMR in *SLC22A5* harbors nine common SNPs, two of which change CG sites. One of these SNPs, rs2631365 A/G is located within 116 bps of CG31, the most proximal CG of the gDMR. The rs2631365 A-allele, which causes loss of a CG site, is associated with higher methylation levels and lower expression of *SLC22A5* RNA. However, our data does not support the idea that gain of a CG causes higher methylation levels within the region.

Correction for variability in blood cell type is required. Therefore, further analysis in Dr. Laprise's laboratory considering differential white blood cells counts was done (Al Tuwaijri et al., 2016). They found that most of these associations in *SLC22A5* was lost following the addition of correction for differential cell counts into the models. Even though, the effect of genotype on DNA methylation level at *SLC22A5* remained significant, the majority of associations between

methylation level and genotype were mostly influenced by variation in eosinophil counts among individuals (Al Tuwaijri et al., 2016). This correlation may be explained by the close linkage between *SLC22A5* and a region that contains a variant associated with eosinophil counts (Martinez et al., 1998) (Gutierrez-Arcelus et al., 2015), which suggests that cell counts may be an essential influencer that affect methylation levels more than genotype in 5q31.

Several studies have reported that DNA methylation could be affected by sex and environmental factors in humans (Kerkel et al., 2008, Robertson, 2005, Harb and Renz, 2015, Naumova et al., 2013). The effect of sex on genetic association in the 5q31 asthmatic region has been documented (Myers et al., 2014). Therefore, we expected to find effects of sex on DNA methylation at genes with allelic expression. Among all tested genes in 5q31, no effect of sex on DNA methylation was found. However, when methylation levels of individual CGs were considered, CG5 in *SLC22A5* showed significantly higher methylation levels in LCL samples from males, although the number of tested samples was small (n=4) for both females and males (Table 3.3). Further investigation with increased sample size is required.

Our study has some limitations which need to be pointed out, due to the small sample sizes of the LCLs and blood that was used in the DNA methylation analysis of the 5q31 region, small genetic effects could be obscured. Additionally, in our DNA methylation analyses we mostly focused on the promoter regions, while gDMRs could also exist in other regulatory elements as enhancers. Another limitation is that studies have shown an influence of immortalized cell lines such as LCLs on methylation level (Saferali et al., 2010, Grafodatskaya et al., 2010), which we tried to avoid by using DNA acquired from blood samples. Because of these limitations, there is a possibility that others gDMRs exist in the 5q31 genomic regions, and require using larger sample sizes.

There was no significant effect of asthma on methylation when all blood samples were considered independent of genotype. However, when the methylation association study was performed to assess the effects of asthma while also including the effects of genotype in the model in Dr. Laprise's laboratory, the significant association between methylation, asthma and genotype in males at the *SLC22A5* gDMR was detected, thus reinforcing that sex is an essential factor that should be considered when the role of DNA methylation is studied.

After we found that DNA methylation in 5q31 may potentially modify the genetically based risk for asthma development, we decided to further characterize the role of DNA methylation in the regulation of *SLC22A5* in the 5q21 asthma-associated region. We tested the effects of the DNA-methyltransferase 1 inhibitor 5-aza-2'-deoxycytidine (5-aza-dC) on DNA methylation of regulatory elements (promoter/exon1) and *SLC22A5* gene expression in HEK293T/17 and NuLi-1 cell lines. We found an upregulation in *SLC22A5* expression levels in both cell lines. However, the promoter region of *SLC22A5* was unmethylated in both the control and the treated cells in HEK293T/17. The upregulation of *SLC22A5* after treatment could be explained by demethylation of another regulatory element that has an influence on *SLC22A5* expression levels. Our results contradict a study by Qu *et al.* (2013) wherein they upregulated *SLC22A5* by using the demethylating reagent, decitabine (DCA), in two cancer lines and observed an increase in the efficiency of *SLC22A5*-mediated uptake of chemotherapeutics. They also found a significant decrease in *SLC22A5* methylation levels post DCA treatment, suggesting an important role for methylation in regulating the expression of *SLC22A5* (Qu et al., 2013). Ramos *et al.* showed that after treatment of HEK293T cells with 5-aza-dC, there were very limited transcriptional changes to demethylation of protein coding genes (Ramos et al., 2015). They proposed that DNA methylation was not a main regulator of the genes' transcriptional activity, although it plays a role in a larger complex system of transcriptional regulation (Ramos et al., 2015).

Thus, the sum of current evidence suggests that the methylation status of the promoter region of *SLC22A5* in 5q31 may act as a potential modifier of the genetically based risk of asthma development. However, DNA methylation is not the only regulator of the *SLC22A5* promoter region in the 5q21 asthma-associated region *in vitro*. It will be interesting to determine the contribution of the discovered association to the development of asthma.

4.2. Sex and genotype influence DNA methylation at the 17q21 locus associated with childhood asthma

In our study, the influence of genotype on methylation levels was tested and validated in several genes in the 17q21 region in LCLs. However, immortalization by the Epstein-Barr virus and long-term cell culture affect DNA methylation patterns (Saferali et al., 2010, Grafodatskaya et al., 2010, Sugawara et al., 2011). Therefore, we studied the association between sex, age and/or genotype and DNA methylation of the 17q21 region using whole blood DNA samples from the

SLSJ familial collection. We indicated that age and sex influence the DNA methylation profiles of the *ZPBP2* proximal promoter region. However, age and sex-specific difference in methylation profiles were small (9% between females and males and 4% between boys and adult males). Our results are consistent with recent findings by Dimas *et al.,* who searched for the expression of quantitative trait loci (eQTLs) in LCLs from females and males and found sex-biased regulatory effects in the *ZPBP2* gene (Dimas et al., 2012). Our data are also consistent with the two recent studies using the Illumina BeadChip array. McCarthy *et al.* performed a comprehensive meta-analysis of 76 studies using a 27K BeadChip array across specimens of multiple tissue types and from adults and children to detect sex-specific methylation patterns in autosomal CpG sites. They found a sex bias towards females in the DNA methylation level of *ZPBP2*, the average differences in methylation levels between females and males were small approximately 4% which is consistent with our result (McCarthy et al., 2014). Another recent study by Yousefi *et al.* that used a 450K BeadChip array across specimens from umbilical cord blood also found that females have a higher methylation level in differently methylated *ZPBP2* regions compared to males; this is the exact same region that was tested in our laboratory (Yousefi et al., 2015). Our findings show that females of any age and adult males have a significantly higher methylation level at the *ZPBP2* proximal promoter. We suggest that one mechanism underlying this is that *ZPBP2* methylation may reduce the genetic association effect in the 17q region and protect females and adult males from asthma development.

The small differences in methylation levels between the sexes in the *ZPBP2* promoter region may reflect larger one in the white blood cell composition of the tested samples, especially since methylation is cell-type specific (Lokk et al., 2014, Jaffe and Irizarry, 2014, Gutierrez-Arcelus et al., 2015). Furthermore, using different types of cell populations that play functionally essential roles in the pathogenesis of asthma (e.g., T-regulatory cells) could increase the differences in DNA methylation levels detected.

Previously, we showed that the *ZPBP2* promoter region also contains an enhancer(s) that acted in cis. We hypothesized that this enhancer(s) may influence the expression level of several neighboring genes (Berlivet et al., 2012). The asthma-associated haplotype HapA is common in population, but not every individual who carry HapA haplotype affected by asthma. This could be due to the influence of differences in environmental exposures, other reason is the inter-individual

variation in DNA methylation found in populations carry asthma risk haplotype HapA. There is a polymorphic SNP in *ZPBP2* intron 5 that harbors a CTCF-binding site (Verlaan et al., 2009). In the Haplotype A, SNP rs1293623 eliminates a CTCF-binding region, while in case of haplotype B a CTCF-binding region exists. We suggested that this SNP that creates CTCF-binding region in *ZPBP2* works as an insulator. Hence, the interaction between the enhancer located within the *ZPBP2* region and the promoters of *ORMDL3, GSDMB* and *GSDMA* was stopped by this insulator. We propose that in the case of the CTCF-binding region (insulator) is being lost, the interactions between distant enhancer in *ZPBP2* and promoters of *ORMDL3, GSDMB* and *GSDMA* genes are permitted on the HapA allele. To understand the role of DNA methylation of the *ZPBP2* promoter in asthma development, we propose that when the *ZPBP2* enhancer region is hypomethylated, the influence of genetic asthma-associated variant is strong, i.e., boys carry the haplotype A. Alternatively, when the *ZPBP2* enhancer region is hypermethylated in females and adult males who carry HapA, it decreases its effect on gene expression, which in turn may protect them from asthma development (Figure 4.1).

In the 17q21 region, we analyzed promoters with allelic bias in their expression levels. We noticed that the 17q21 asthma-associated region has two gene promoters: *ZPBP2* and *GSDMA*, which contain gDMRs (Al Tuwaijri et al., 2016). Strikingly, genes that showed the strongest bias in allelic expression in LCLs and CD4+ lymphocytes such as *ORMDL3* and *GSDMB* were not indicated (Ge et al., 2009, Murphy et al., 2010, Verlaan et al., 2009). This is consistent with other study, that found asthmatic children have significantly higher *ORMDL3* methylation levels compared to controls, and the methylation levels was correlated with *ORMDL3* expression in leukocytes. However, the polymorphism in this region and CpG methylation were independently associated with *ORMDL3* expression suggesting, two different mechanisms modulating gene expression (Acevedo et al., 2015). Furthermore, our results are in agreement with other study that found SNPs near to *GSDMA* promoter influence DNA methylation levels (Liu et al., 2014). The influence of genotype on DNA methylation levels in *ZPBP2* and *GSDMA* at differential blood cell counts were included in the model in Dr. Laprise's Laboratory, and they found the effect of genotype on methylation remained significant in both *ZPBP2* and *GSDMA* (Al Tuwaijri et al., 2016). This could be due to the *GSDMA* location within a region associated with white blood cell counts and neutrophil counts (Okada et al., 2010, Crosslin et al., 2012). To understand the interaction between genotype, DNA methylation, asthma and white blood cell counts in these

regions, using purified blood cell samples in DNA methylation study would be recommended (Martino et al., 2014, Liang et al., 2015).

We found in both *ZPBP2* and *GSDMA* gDMRs, the asthma-associated HapA samples have a dominant effect on methylation levels. The methylation levels in heterozygous samples showed methylation status similar to those found in samples that are homozygous for the HapA (Al Tuwaijri et al., 2016). It is worth noting that the distance between *ZPBP2* and *GSDMA* is 95 kb, such a similarity in the dominant effect of genotype on methylation may be extended and cover larger region, affecting several CGs and possibly leading to general increase in HapA homozygotes and heterozygotes expression. Indeed, study used cord blood mononuclear cells, showed that risk polymorphisms for asthma in the 17q21 locus were strongly associated with increased *GSDMA* gene expression levels (Lluis et al., 2011), whereas *ZPBP2* RNA abundance was found to be inversely correlated with DNA methylation in LCLs in our lab (Berlivet et al., 2012). In general, these findings lead us to expect that the expression levels of both genes *ZPBP2* and *GSDMA* increased in the blood cells of homozygous HapA and heterozygous samples, which needs to be investigated in future studies (Al Tuwaijri et al., 2016).

When we investigated the association between DNA methylation levels in 17q21 region and asthma including genotype, we found sex bias with significant association between methylation, asthma and genotype in females at the *GSDMA* and *ZPBP2* gDMRs. In general, these results highlight the essential role of sex in DNA methylation, which should be considered and accounted for in studies.

Epigenetic factors such as DNA methylation play a fundamental role in regulating gene expression, although the mechanism remains elusive (Wagner et al., 2014). Here, we indicated the effect of changes in DNA methylation on expression levels and allele-specific expression (ASE) among genes in the asthma-associated region, 17q21. Apparently, a modest decrease in average promoter methylation levels caused a big increase in expression levels of *ZPBP2* and *GSDMA* genes following demethylation with 5-aza-dC treatment (4- to 20-fold increase). This may explain the differences in promoter methylation levels that were found between asthmatic and non-asthmatic females. Moreover, we noticed that genes that were not expressed in control cells remained silent after 5-aza-dC treatment, suggesting that the efficiency of the 5-aza-dC used in the experiment is limited when the target genes are located in regions of DNA that are heavily

methylated. We suggested that different genes in the same 17q21 region might respond in different ways to loss or gain of methylation. To investigate if DNA methylation was responsible for the influence of genotype on gene expression levels, the allelic expression of 17q21 genes was evaluated pre- and post- 5-aza-dC treatment. We found two genes in the same 17q21 region that acted differently after 5-aza-dC treatment and showed different allelic expression levels. The *ZPBP2* allelic imbalance favored the expression of HapB and reactivated the HapA allele. Also, it is important to notice that of the genes we tested, *ZPBP2* is the only gene in the region that showed correlation between allelic methylation and allelic bias in expression (Figure 3.14A). The *ORMDL3* promoter cannot be demethylated by 5-aza-dC because it was hypomethylated in both HEK293T/17 and NuLi-1 cell lines, although we observed a slight increase in *ORMDL3* expression in HEK293T/17 after 5-aza-dC treatment (Figure 3.13B). Interestingly, the *ORMDL3* allelic difference in expression was changed from the HapA towards the HapB allele (Figure 3.13C). The other two genes that showed allelic differences in expression were *IKZF3* and *GSDMA,* which sustained their allelic bias with generally upregulation in their expression following treatment with 5-aza-dC (Figure 3.13B). Allele-specific analyses of gene regulation point to genetic polymorphisms that play a fundamental role in changing transcription factor binding sites and are involved in specific chromatin mechanisms (Verlaan et al., 2009) (Pastinen et al., 2004). We concluded that DNA methylation plays an important role in allelic expression for some non-imprinted genes, and our results are in agreement with other studies that suggested a complex and elusive relationship between DNA methylation and gene expression (Wagner et al., 2014)(reviewed in (Do et al., 2017)). Our results are in accordance with a study that has been done with different human tissues and cell types. They found that most DMRs are restricted to cell type ASM, and some of them exist in a GWAS region associated with immunological disorders (Do et al., 2016). The same study as well as others proposed the important role of the polymorphic CTCF-binding site and other transcriptional factors in allelic methylation asymmetry (Do et al., 2016) (Paliwal et al., 2013). It would therefore be interesting to test if the CTCF-binding site located in 17q21 has an influence on the allelic methylation and expression imbalance.

One caveat in our study is that, while these data are based on the analysis of two cell lines, only Nuli-1 is heterozygous. Therefore, we need confirmation in a larger sample, especially since one study reports that the haplotype ASM is tissue and cell specific (Do et al., 2016). Moreover,

we have to consider that the *ZPBP2* promoter has lower methylation levels in males (Naumova et al., 2013), hence the analysis of ASM and ASE would be more informative in heterozygous males.

In summary, our findings are the first that show *ZPBP2* sex-specific, age, and genotype-dependent that influence DNA methylation in a GWAS locus in 17q21 which is consistent with the nature of asthma as more boys than girls develop asthma during childhood, with the ratio shifting during adulthood. Our data supports our main hypothesis that variation in DNA methylation at regulatory elements within asthma-associated genomic regions 17q21 acts as a modifier of the effect of genotype on phenotype. Our results suggest that the relationship between DNA methylation and allelic expression is variable for different genes in the same region. *ZPBP2* seems to be an unexpected candidate as a causal gene for asthma and autoimmune disease due to its known role in sperms' interaction with the oocyte's *zona pellucida* during fertilization. However, the fact is that it is expressed in somatic cells (LCLs and NuLi-1), and it could have an elusive function that needs to be explored. Additionally, a recent study indicated that rare mutations in *ZPBP2* have been found in pediatric patients with inflammatory bowel disease and asthma (Andreoletti et al., 2015). In our experiments, two cell lines were used, NuLi-1 and HEK293T/17, both reacted differently to 5-aza-dC (decitabine), a chemical that is prescribed to MDS patients, suggesting that even with providing the same environmental conditions, levels of DNA methylation and gene expression responses are different between different cell types. Also, it is important to mention that both NuLi-1 and HEK293T/17 have multiple chromosomal numbers, both cell lines derived from different sexes, NuLi-1 cells are derived from a normal lung male donor, while HEK293T/17 are derived from a female donor. Thus, karyotype, sex, and cell type are all factors that need to be considered in demethylation experiments.

4.3. X chromosome dosage and presence of sex-determining region Y (*SRY*) may cause epigenetic sex differences in asthma region 17q21

We demonstrated that a single regulatory region on the *ZPBP2* promoter showed statistically significantly higher DNA methylation among females compared to males in whole blood (Naumova et al., 2013). We expected that the X chromosome dosage might influence the DNA methylation levels at the proximal *ZPBP2* promoter region flanking the TSS. In agreement with our hypothesis, our results demonstrated that DNA methylation levels at the *ZPBP2* promoter region in fibroblast cell lines correlate with the number of X chromosomes regardless of sex

phenotype. Interestingly, we also noticed that when *SRY* was present, the methylation levels in fibroblast cell lines with more than one dose of the X chromosome were decreased, suggesting a possible protective role that was exerted by the *SRY* against gene silencing at the proximal *ZPBP2* promoter region (Figure 3.15D). Our results are in agreement with several studies that suggest the influence of sex chromosome dosage on differences in DNA methylation and gene expression is specific to autosomal loci (Ober et al., 2008, Grafodatskaya et al., 2013, Arnold et al., 2012, Hall et al., 2014, Arnold et al., 2016, Liu et al., 2010a, Sharma et al., 2015). They are also consistent with the results of others that show statistically significant increases in DNA methylation levels in the CGI of specific autosomal loci with increased X chromosome dosage (Grafodatskaya et al., 2013). A more recent study assessed the expression of miRNAs and autosomal genes in addition to the DNA methylation levels in untransformed human fibroblast cells derived from individuals with different sex chromosome dosages: 45, X, 46, XX and 47, XXX. Consistent with our results, they observed in a set of autosomal genes such as Claudin 11 (*CLDN11*) that the methylation of the promoter region was associated with the X chromosome dosage and inversely correlated to *CLDN11* expression. Interestingly, they found that maintenance of *DNMT1* gene expression was highest in 47,XXX and decreased in 46,XX, followed by 45,X (Rajpathak and Deobagkar, 2017). Another recent genome-wide methylation analysis has been conducted using methylated DNA immunoprecipitation (MeDIP) and 27K Illumina's Infinium assay to compare DNA methylation levels of CGI and promoters in blood samples derived from patients with Turner syndrome (45,X) and Klinefelter syndrome (47,XXY) to normal patients, 46,XX and 46,XY. In agreement with our findings, they showed that Turner individuals lost autosomal DNA methylation about five times more than Klinefelter individuals. Interestingly, they also found that *ZPBP2* was hypomethylated in Turner individuals compared to normal females, whereas no difference in methylation was detected in Turner individuals compared to normal males, suggesting that one X was associated with hypomethylation on the autosomes and, consistent with our findings, that an increased number of X chromosome copies is associated with higher methylation levels in the autosomal region (Sharma et al., 2015). Contrary to our results, Sharma *et al.* (2015) did not find an effect of the *SRY* gene on *ZPBP2* methylation when they compared 47,XXY to normal females 46,XX in whole blood, supporting the notion that sex-specific DNA methylation levels may change between different cell types (Sharma et al., 2015, Eckhardt et al., 2006). Trolle *et al.* performed a genome-wide DNA methylation and expression analysis using high-resolution 450K-Illumina Infinium

assay and RNA-seq in the leukocytes of individuals with Turner syndrome (45,X) and compared to normal 46,XX and 46,XY controls. They found females with Turner syndrome (45,X) were commonly have lower global DNA methylation levels that were not limited to proximal promotors in autosomal regions and in DMR, but extended to repetitive elements as well compared to 46,XX females. Importantly, the global DNA methylation levels in 45,X was higher than in 46,XY males (Trolle et al., 2016).

Four Core Genotypes (FCG) is one example of several approaches used to reveal the origins of sex differences and to determine whether they are due to a sex complement mechanism or gonadal hormones. In our study, we included fibroblast cell lines derived from 46,XY sex reversal SRY female patients, 46,XX male syndrome, Turner 45,X and other aneuploidy samples. We found that *ZPBP2* DNA methylation increased in samples with more than one X chromosome in the absence of *SRY*. In one study, the FCG model was used to analyze genome-wide gene silencing in T lymphocytes. They found the expression of a group of autosomal sex-chromosome-complement-sensitive (SCS) genes was affected by the presence of the *Sry* gene, and they noticed that several genes sensitive to the sex chromosome complement phenomenon were increased in expression in XY^{-} females compared to XY^{-}*Sry* males. This suggests that *Sry* may contribute in global gene expression and has a repressive influence in a sex chromosome complement-specific manner (Wijchers et al., 2010). Our results are in agreement with the interconnection between the sex chromosome dosage and *SRY*. However, our results contradict the Wijchers *et al.* repressive influence of *Sry*, and we believe that *Sry* has a protective role against *ZPBP2* silencing in cell lines with more than one X chromosome.

Several studies discussed the essential role of non-gonadal mechanisms (sex chromosome dosage) to explain the sex bias in autosomal gene expression between males and females in somatic cells. However, the mechanism in how sex chromosomes lead to epigenetic sex bias in non-hormonal manners is still obscure. Indeed, it has been indicated that *SRY* may have functions other than the classic gonad-specific transcription factor and testis determination. *Sry* has been found to be expressed in the substantia nigra of the adult male rat. Knocking down *Sry* in male rats caused downregulation of tyrosine hydroxylase (TH) expression, the rate-limiting enzyme in the dopamine synthesis mechanism, leading to motor dysfunction. Given the fact that females do not show overt motor dysfunction due to the absence of *Sry, Sry* may compensate for a female-specific

factor that is essential for TH expression maintenance in the neurons of the substantia nigra (Dewing et al., 2006). In humans, *SRY* is expressed in the hypothalamus and cortex of the adult male brain (Mayer et al., 1998). Czech *et al.* found *SRY* transcripts localized in substantia nigra pars compacta within TH-positive neurons. Knockdown *SRY* in a human neuroblastoma cell line caused reduction in TH expression. Overexpression of *SRY* caused higher TH expression, which lead to increased extracellular dopamine levels. This suggests that abnormal *SRY* expression may explain why increased dopamine disorders such as Parkinson's disease or schizophrenia are more common in males (Czech et al., 2012). It has been indicated that *SRY* regulates the expression of another neural enzyme, monoamine oxidase A (MAO A), located in the X chromosome in neuroblastoma cell lines. MAO A plays an essential role in brain development and function, and dysregulation in MAO A has been indicated in several psychiatric disorders that show sexual dimorphism in disease pathogenesis, like autism and attention deficit hyperactivity disorder (Wu et al., 2009). The mechanism in how the *SRY* involved in DNA methylation of autosomal region such as *ZPBP2* is not fully understand. Nevertheless, it is important to mention that *ZPBP2* is highly expressed in testes and necessary for fertilization (Lin et al., 2007). We suggest that *SRY* interacts with *ZPBP2* and protects it from the DNA methylation effects, this could explain our previous findings that females showed higher DNA methylation levels in the *ZPBP2* promoter region compared to males. Our results are consistent with other published studies that have suggested an effect of sex chromosome complement in the sex bias in autoimmune disease. It has been indicated that almost 80% of autoimmune patients are females. In a study using the FCG model to investigate whether sex chromosome complement may be associated with the sex differences in susceptibility to experimental autoimmune encephalomyelitis (EAE) and lupus, they found that XX mice were more susceptible to EAE and lupus than XY^- females. The production of Th2 cytokines (IL13, IL4, and IL10), which play an essential role in the protection against disease in EAE, was higher in XY^- mice compared to XX mice (Cetean et al., 2015). In a study by Scofield *et al.,* they showed that the prevalence of SLE in 47,XXY Klinefelter syndrome was approximately 14-fold higher compared to normal males 46,XY. This risk reflects the increased female risk to having SLE, which is also about 14-fold compared to males (Scofield et al., 2008). Another recent study reported that the prevalence of autoimmune diseases such as SLE and Sjogren's syndrome (SS) was increased approximately 2.5-2.9-fold in 47,XXX females compared

to 46,XX normal females, and 25-41-fold compared to 46,XY normal males. Both previous studies support the idea of an effect of the X chromosome on gene dose (Liu et al., 2016).

We found variability in methylation levels at *ZPBP2* in individuals who carried more than one X (Figure 3.15C). This variation could be attributed to age; the sex of the donor of the fibroblast cell lines (Saferali et al., 2010, Boks et al., 2009, Naumova et al., 2013); the number of passages of cell lines(Grafodatskaya et al., 2010); environmental factors (Czyz et al., 2012), X-linked genes that escape X-inactivation(Wijchers and Festenstein, 2011); or a stochastic effect. Some of these possible causes were tested. The association between age and DNA methylation differences has been indicated at several CpG sites in human fibroblasts (Koch et al., 2011). Interestingly, our findings showed there was a moderate correlation between the age of the donor who carried more than one X and the variability in methylation levels at the *ZPBP2* gene. These results are consistent with our previous findings that age influences DNA methylation patterns in the same promoter region of the *ZPBP2* gene we tested (Naumova et al., 2013). Other studies reported that DNA methylation changed through long-term cell culture and was affected by the age of the donors of human mesenchymal stromal cells (Bork et al., 2010).

We found the effect of the X chromosome complement on methylation of *ZPBP2* was stronger in samples with more than one X chromosome. We hypothesized that one or more X-linked genes is responsible for these differences in DNA methylation. The effect of the X-linked *MECP2* mutations on *ZPBP2* methylation was assessed. MeCP2 is a nuclear protein that does not escape XCI and is well known as a transcriptional regulator and chromatin remodeling protein that plays an essential role in controlling gene expression through recognizing methylated DNA via its methyl-CpG–binding domain (MBD) (Nan et al., 1998, Adler et al., 1995, Hansen et al., 2010). It has been shown that *MECP2* acts as a methylation-specific transcriptional repressor that interacts with chromatin modifying complexes, HDAC and sin3A, to repress transcription (Jones et al., 1998). Interestingly, we found that fibroblast cell lines with more than one X lose their differential methylation in Rett syndrome females with *MECP2* mutations. Given the small sample size in this pilot experiment, which consisted of three fibroblast cell lines, further study with more *MECP2*-mutation cell lines should be included in order to assess the potential effect of *MECP2* on sex-specific *ZPBP2* DNA methylation.

We suggested that X-linked genes that escape X-inactivation could also account for some of the sex-specific alterations in DNA methylation we observed in *ZPBP2*. In humans, about 15% of X-linked genes escape XCI, most of them enriched in Xp, the short arm of the X chromosome (Carrel and Willard, 2005, Peeters et al., 2014, Berletch et al., 2010, Cotton et al., 2011). Cotton *et al.* analyzed SNP allelic dosage to characterize genes that escape X-chromosome inactivation and are expressed by both X chromosomes. They considered the Xi gene that expressed at threshold of 10% compared to that noticed from active X as escape genes. Some of the genes from the inactive X chromosome continue to be expressed. 13% of observed X-linked genes escaped, and 29% were considered variably escaped in some but not all females (Cotton et al., 2013), suggesting that escaping from XCI will not only cause variability in a gene's expression between both sexes, but also cause differences in a gene's expression between tissues in the same female (Carrel and Willard, 2005) (Cotton et al., 2011).

These genes have been shown to have sex-specific differences in traits, including susceptibility to disease (Ober et al., 2008) (Arnold et al., 2012). We suggest that a gene or genes escaping XCI may influence methylation levels of regulatory elements located on autosomes, *KDM5C* was selected to be tested in a pilot experiment. The X-linked gene *KDM5C* escapes XCI and encodes an enzyme that demethylates H3K4me3/me2 (Iwase et al., 2007). According to the literature, the most plausible mechanism is in active promoter regions; Histone H3K4 is di- and trimethylated. Methylated H3K4 prevents *de novo* DNA methyltransferase DNMT*3A* from accessing the promoter region. *DNMT3A* cannot methylate the CGs in the promoter region and, therefore, the gene is active. When it demethylates H3K4me3/me2, it allows *DNMT3A* to access the CGs in the promoter. This causes methylation of the promoter region and silencing of the gene (Iwase et al., 2007, Otani et al., 2009, Grafodatskaya et al., 2013). For that reason, the role of the best candidate gene, *KDM5C,* was tested by depletion of *KDM5C* RNA using siRNA in HEK293T/17 cell lines. The DNA methylation profile of the *ZPBP2* promoter was measured to investigate if *KDM5C* plays a role in maintaining differences in DNA methylation levels between males and females. Individuals that have a higher number of X chromosomes would have higher levels of *KDM5C* in their cells, which would increase the global *KDM5C* activity (Grafodatskaya et al., 2013). Knocking down the X-linked *KDM5C* gene in the HEK293T/17 cell line did not show a significant effect on overall *ZPBP2* promoter methylation levels. It also did not affect the expression levels of *DNMT1* or *DNMT3A*, but increased the expression of *IKZF3,* which is located

very close to *ZPBP2* (about 4 kb). Previously, we hypothesized that the *ZPBP2* enhancer region exerts a long-range regulatory effect that acts distally to regulate the expression of neighboring genes (Verlaan et al., 2009, Berlivet et al., 2012). It has been indicated that *KDM5C* can be recruited in both promoter and enhancer regions through gene-specific transcriptional factors and acts as both a repressor of promoter activity and an activator of enhancer activity (Outchkourov et al., 2013). This could explain why knockdown of *KDM5C* did not influence *ZPBP2* directly, but affected the adjacent gene, *IKZF3*. Furthermore, when we transfected HEK293T/17 with siKDM5C, the KDM5C was downregulated ~75%. We did not assess the knockdown efficiency (at the protein level) by using e.g. western blot. If *KDM5C* has high stability with high half-life, or if it has another isoform that was not recognized by siRNA (Kisielow et al., 2002), we would see no effect in protein levels even though *KDM5C* mRNA was reduced. Assessing sensitivity and specificity of siKDM5C using the luciferase reporter system to test the efficacy of siRNA is recommended in the future.

We concluded that *KDM5C* did not influence the sex-specific methylation levels of the regulatory element located in the *ZPBP2* promoter region. This could be for several reasons: (1) There is no relationship between *KDM5C* and the difference in methylation levels of *ZPBP2* between males and females; (2) *KDM5C* influences are a part of the X-linked gene network, synergized to mediate sex bias DNA methylation in the autosomal region; (3) *KDM5C* is important for establishing sex-specificity in *ZPBP2* DNA methylation early in development (*de novo* DNA methylation) and is not related to the maintenance of DNA methylation; (4) *KDM5C* demethylates the active promoter and TSS H3K4me3 mark, but not the active enhancer mark H3K4me1, which is also enriched in downstream TSS. This later possibility may explain why knockdown of *KDM5C* did not change methylation levels in the *ZPBP2* promoter region. The contribution of *KDM5C* to sex bias in DNA methylation patterns at the *ZPBP2* proximal promoter region remains inconclusive.

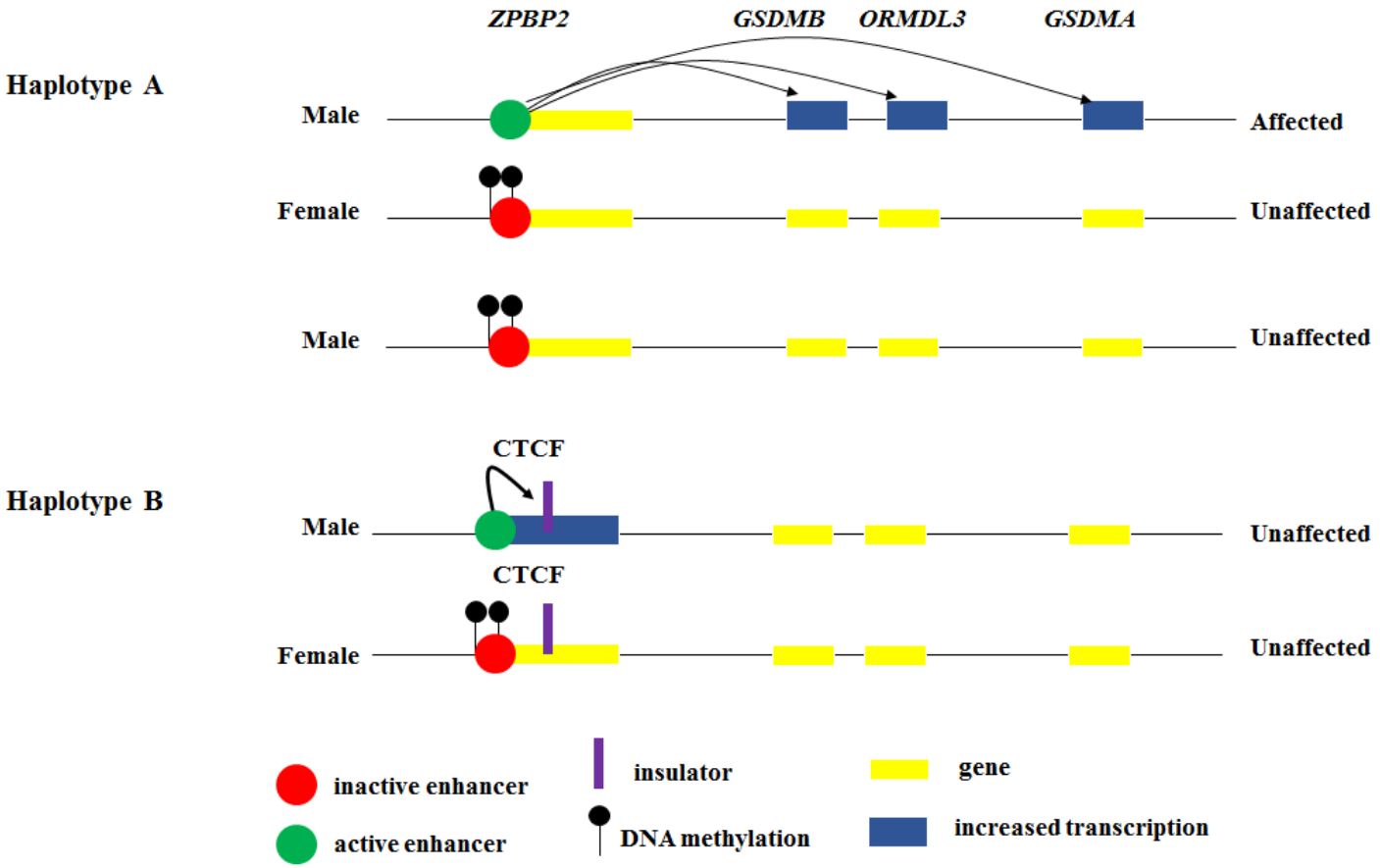
In humans, differences in DNA methylation have been suggested to be involved in complex diseases, including asthma predisposition (Kabesch et al., 2010). Sexual dimorphism of DNA methylation in the autosomal region has been reported in several studies, including *ZPBP2* in the asthma region of 17q21(Boks et al., 2009) (Liu et al., 2010a) (Hall et al., 2014, Sawalha et al., 2012, McCarthy et al., 2014, Yousefi et al., 2015) (Naumova et al., 2013). However, sex-specific

DNA methylation in the autosomal region due to X chromosome dosage has not been investigated in these studies. In this pilot study, we demonstrate that the X chromosome dose and SRY influence the DNA methylation level at the *ZPBP2* autosomal region. There are some limitations to this study. We used human DNA from untransformed fibroblast cell lines that were acquired from individuals carrying different karyotypes of sex chromosome aneuploidy and mutations, including sex reversal, Rett syndrome and Turner's syndrome. The DNA was extracted from fibroblast cell lines with different passage numbers (2-16) (Table 2.4). It has been suggested that cell culture and multiple passages may contribute to DNA methylation differences in fibroblasts, which explains the variability in our DNA methylation analysis from fibroblast cell lines (Ivanov et al., 2016). Hence, using patient samples is recommended in future work.

Our results indicate the influence that X-chromosome dosage and SRY has on sex-specific *ZPBP2* DNA methylation. DNA samples derived from Rett syndrome patients with *MECP2* mutations reveal that *ZPBP2* reduces methylation levels compared to normal 46,XX females. Further analysis of blood samples is recommended to confirm our findings on fibroblast cell lines and to map the exact region of X chromosomes that are responsible for the sex bias in DNA methylation in autosomal region.

FIGURES

Figure 4.1. Pictorial depiction described association between genetic polymorphism and differences in DNA methylation in the 17q21 region. In 17q21 region, there are two common haplotypes, haplotype A (with asthma associated alleles) and haplotype B (with non-asthma associated alleles), both demonstrated association with expression levels of *ORMDL3*, *GSDMB*, *GSDMA* and *ZPBP2*. Haplotype A is associated with up-regulation in *ORMDL3*, *GSDMB* and *GSDMA* expression levels, but lower expression of *ZPBP2*, whereas, Haplotype B is associated with down-regulation in *ORMDL3*, *GSDMB* and *GSDMA* expression levels and increase in *ZPBP2* expression. In the Haplotype A, a CTCF-binding site is abolished by SNP rs12936231, while it exists in the haplotype B. We hypothesized that this polymorphic CTCF-binding region acts as an insulator in *ZPBP2*, preventing interaction between the enhancer located within the *ZPBP2* region and the promoters of *ORMDL3*, *GSDMB* and *GSDMA*. In haplotype A allele, when CTCF-binding site is lost, the interactions between distant enhancer in *ZPBP2* and promoters in *ORMDL3*, *GSDMB* and *GSDMA* is allowed. When the *ZPBP2* enhancer region is hypermethylated in females and adult males, including individuals who carry haplotype A, it attenuates its effect on *ORMDL3*, *GSDMB* and *GSDMA* gene expression, which in turn may protect from asthma development. As increased expression of in *ORMDL3, GSDMB* and *GSDMA* is suspected to increase the risk of childhood asthma, *ZPBP2* methylation may be reducing the risk (Naumova et al., 2013).


Haplotype A
ZPBP2
GSDMB
ORMDL3
GSDMA
Male
Affected
Female
Unaffected
Male
Unaffected
Haplotype B
CTCF
Male
Unaffected
CTCF
Female
Unaffected
inactive enhancer
active enhancer
insulator
DNA methylation
gene
increased transcription

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

5.1. Conclusions

- Genotype influences DNA methylation levels at the *SLC22A5* gene promoter region in DNA samples from LCLs and peripheral blood, and inversely correlates with the differences in allelic expression on *SLC22A5*, which is consistent with our hypothesis.
- No significant effect of sex on methylation levels was detected in LCLs in 5q31 region.
- Demethylation of HEK239T/17 using 5-aza-dC treatment caused upregulation in *SLC22A5* expression level but did not change its promoter methylation level, suggests the possibility of involvement of another regulatory region.
- Genotype influences DNA methylation levels at the *GSDMA* and *ZPBP2* promoter region in DNA samples from peripheral blood and has a dominant effect on *ZPBP2* and *GSDMA* methylation with lower methylation levels in individuals that carry the asthma-predisposing alleles HapA.
- *ZPBP2* promoter region showed inter-individual variation in methylation in a sex-specific manner with significantly higher methylation in females.
- A modest decrease in average promoter methylation levels caused a big increase in expression levels of *ZPBP2* and *GSDMA* genes following demethylation with 5-aza-dC treatment.
- There was a modification in allelic expression levels of *ZPBP2* and *ORMDL3* after 5-aza-dC treatment, suggesting that each allele may act differently to methylation changes.
- Sex bias in DNA methylation levels in *ZPBP2* promoter is influenced by *SRY* and X chromosome dosage but not in sex phenotype in fibroblast cell lines.
- DNA samples derived from Rett syndrome patients with *MECP2* mutations showed lower methylation levels compared to normal 46,XX females.

5.2. Future directions

5.2.1. Map the region of the X chromosome that could be responsible for sex-specific DNA methylation in the autosomal genetic regions

Sexual dimorphism in DNA methylation could result from hormonal differences or the sex chromosome dosage (Arnold et al., 2012). We found that *ZPBP2* DNA methylation levels were higher in females than males due to dosage of the X chromosome rather than the sex hormone level. Although there are many studies about sex-specific DNA methylation, the mechanism remains elusive. There are other autosomal regions that showed sex bias in DNA methylation, where females have higher methylation levels. We hypothesize that the sex bias in DNA methylation levels in several autosomal regions is caused by sex chromosome dosage, with this bias resulting from X-linked genes that escape X chromosome inactivation and are involved in the interplay between histone modifications, DNA methylation, and transcriptional regulators. A related study by McCarthy *et al.* involved a meta-analysis of 76 other studies all using the 27 K BeadChip array to assess sex bias in DNA methylation in autosomal genetic regions using blood samples (McCarthy et al., 2014). We may pursue our study by including other autosomal regions that show >5% higher DNA methylation levels in females and regions that were found by McCarthy *et al.* (Table 5.1). We may first start by comparing the DNA methylation in regions involving CGs showing sex-specific DNA methylation between normal males and females (Table 5.1) using sodium bisulfite sequencing methylation assays followed by pyrosequencing methylation analysis in fibroblast cell lines to validate the results of McCarthy *et al.* in fibroblast cell lines. After we confirm the sex bias in all or some of previous regions (Table 5.1), then we will assess whether the sex-specific DNA methylation is caused by sex chromosome dosage or sex phenotype. Sodium bisulfite sequencing methylation assays will be conducted using DNA samples extracted from untransformed human fibroblast cell lines derived from individuals with different sex chromosome dosages: 46, XY females (sex reversal); 45, X females (Turner syndrome); 46, XY males; 46, XX females; 47, XXX females; 46, XX males; 47, XXY males Klinefelter syndrome; and a 49, XXXXY male. We expect that an increase in DNA methylation levels in these regions will be associated with X chromosome dosage regardless of sex phenotype.

To map the region in the X chromosome that could be responsible for sexual dimorphism in DNA methylation in the confirmed autosomal regions, we would need to conduct Sodium

bisulfite pyrosequencing or sodium bisulfite sequencing methylation assays in the validated autosomal regions using DNA samples from fibroblast cells with different X chromosome rearrangements (translocations, duplications and deletions) and mutations in X-linked genes (such as RETT syndrome *MECP2* mutations, XLID syndrome *KDM5C* mutations and ATRX syndrome *ATRX* mutations). After we narrow down the potential target genes, then we can investigate the genes that escape X inactivation and may possibly influence the sex-specific DNA methylation in autosomal regions. We could then confirm our findings in fibroblast cell lines by using blood samples from patients with different X chromosome rearrangements.

5.2.2. Start a pilot experiment using a candidate gene to knock down the X-linked *KDM6A* gene that may cause sex differences in DNA methylation in the NuLi-1 cell line

Although X inactivation is thought to equalize differences in gene expression between males and females, there are some genes that escape XCI and are potentially associated with differences between sexes (Cotton et al., 2013). Zinn *et al.* determined that haploinsufficiency of one or more X-linked genes in Xp22.3, which escape XCI, are responsible for the Turner patients' neurocognitive phenotype, involving impaired visual-spatial abilities (Zinn et al., 2007). There is a possibility that gene(s) that escape XCI also contribute to sex-specific DNA methylation patterns. *KDM6A* was selected because it is an X-linked gene that escapes XCI and encodes an enzyme that preferentially demethylates di-methylated and tri-methylated forms of lysine 27 on histone H3, a mark of inactive chromatin. Methylation of H3K27 is considered to be an essential factor of transcriptional gene silencing, and thus, *KDM6A* plays an important role in activation of gene expression. *KDM6C* is a Y-linked homologue for *KDM6A* in humans and mice. Xu *et al.* used the FCG mouse model to investigate whether the increased expression of *Kdm6a* was dependent on X chromosome dosage, and not on sex phenotype. They found that female mice had higher expression levels of *Kdm6a* than males in specific brain regions, such as the cortex and hippocampus, and that this sex-specific difference in *Kdm6a* expression depends on the sex chromosome complement rather than the gonadal sex of the mice (Xu et al., 2008b). Additionally, mutations in *KDM6A* were identified in patients with a genetic disease called the Kabuki syndrome. Furthermore, *KDM6A* is considered to be a tumor suppressor that is involved in several types of cancer, such as myeloma and renal cancer (reviewed in (Van der Meulen et al., 2014)).

We may start with a pilot study and use a candidate gene approach to test the possibility that *KDM6A* plays a role in the maintenance of sex-specific differences in methylation levels at the *ZPBP2* promoter. *KDM6A* will be depleted in the NuLi-1 cell line using *KDM6A*-specific siRNA or small hairpin RNA (shRNA). We expect that the depletion of the X-linked *KDM6A* gene in NuLi-1 would decrease expression of *ZPBP2* and increase the DNA methylation at its promoter. Expression and methylation of *ZPBP2* will be assessed in the transfected NuLi-1 cell line and in controls.

TABLES

Table 5.1 Some of selected autosomal regions showing >5% sex-specific DNA methylation (McCarthy et al., 2014).

Gene	*Chr*	*Target ID*	*Female, average Beta*	*Male, average Beta*	*Meta-analysis P Value*	*NCBI description of protein function*	*Genome-wide association study traits*
ZPBP2	17	cg05330360	0.74	0.67	<2.2E-16	Is implicated in sperm-oocyte interaction during fertilization.	Childhood asthma, Crohn's disease, ulcerative colitis, Type 1 diabetes, biliary cirrhosis and rheumatoid arthritis
SLC9A2	2	cg20050113	0.47	0.38	<2.2E-16	Involved in pH regulation to eliminate acids generated by active metabolism or to counter adverse environmental conditions.	Obsessive-compulsive disorder and anxiety
DDX43	6	cg08124399	0.75	0.67	<2.2E-16	ATP-dependent RNA helicase in the DEAD-box family.	N/A
SPESP1	15	cg09886641	0.74	0.67	<2.2E-16	Human alloantigen involved in sperm-egg binding and fusion.	N/A
FIGNL1	7	cg05072008	0.49	0.42	<2.2E-16	May regulate osteoblast proliferation and differentiation.	Crohn's disease and Acute lymphoblastic leukemia
CRISP2	6	cg04595372	0.62	0.56	3.82E-13	Also, known as Testis-Specific Protein TPX-1. May regulate some ion channels' activity and thereby regulate calcium fluxes during sperm capacitation.	Osteoporosis
NUPL1	13	cg08532057	0.26	0.21	<2.2E-16	Component of the nuclear pore complex, a complex required for the trafficking across the nuclear membrane.	N/A

Table 5.2 *KDM6A* location, percentage of females which escape from XCI, genetic status and function (Cotton et al., 2013).

Gene name	***Location***	***Homolog on the Y***	***Percentage of females which escape from XCI***	***Genic XCI status***	***Average %Xi expression***	***Function***
KDM6A (UTX)	Xp11.2	UTY	65%	variable escape	49%	Catalyze the demethylation of tri/di-methylated lysine 27 of histone H3

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LIST OF WEBSITES

- Primer3 software (v. 0.4.0) http//bioinfo.ut.ee/primer3-0.4.0
- UCSC Genome Browser https://genome.ucsc.edu
- Gtexportal https://www.gtexportal.org/home/
- Quma http://quma.cdb.riken.jp/

LIST OF SOFTWARE

- BioEdit
- BiQ Analyzer

APPENDIX

A.1 Association between asthma and *SLC22A5* methylation levels in DNA samples from peripheral blood

Dr. Laprise lab conducted pyrosequencing assays and analysis and did not find any significant effect of asthma on methylation in any of the seven CGs in *SLC22A5* (from p = 3.1E-01 to p = 6.7E-01) in DNA samples from peripheral blood when all individuals were included, which was independent of the genotype. However, when they included the effect of genotype and asthma on DNA methylation, they noticed significant differences only for males (from p = 1.2E-04 to p = 5.5E-05). Due to small sample size for individuals younger than 16 years, the effect of age couldn't be assessed (Al Tuwaijri et al., 2016).

A.2 Sex influences DNA methylation levels at the *ZPBP2* promoter region in DNA samples from peripheral blood using pyrosequencing methylation assay

The sex-specificity of the *ZPBP2* promoter DNA methylation results were validated using pyrosequencing methylation assay in Dr. Laprise's laboratory, 11 CGs homozygous for the HapA haplotype from the *ZPBP2* promoter region, all located in the transcriptional start site were analyzed. *ZPBP2* DNA methylation was assessed in 18 males and 19 females that were younger than 16 years (they selected the age threshold based on Moffatt *et al*. (Moffatt et al., 2010). According to the pyrosequencing assay analysis, they confirmed our findings of higher methylation level in females (35%) compared to males (26%) at all 11 tested CG sites (t-test statistics, p = 6.65E-10) (Naumova et al., 2013).

A.3 DNA methylation levels increase with age in males at the *ZPBP2* promoter region in DNA samples from peripheral blood using pyrosequencing methylation assay

To investigate if DNA methylation levels of the *ZPBP2* promoter possibly increased with age. Average DNA methylation levels were tested and analyzed across 11 CG sites in 18 males younger than 16 (age range 5–16) and 43 adult males (age range 19–80) using the pyrosequencing methylation assay in Dr. Laprise's laboratory. They found that the average *ZPBP2* methylation level in adult men (30%) was significantly higher than in young boys (26%) (t-test statistics, p

=1.2E-03), suggesting that the increase of DNA methylation at the *ZPBP2* promoter region in adult males may play a protective role while also reducing the role of the regulatory element in asthma predisposition (Naumova et al., 2013).

A.4 Association between *ZPBP2* and *GSDMA* methylation levels and asthma

DNA methylation levels of *ZPBP2* and *GSDMA* were compared between asthmatic and non-asthmatic samples, with respect to genotypes and WBC counts which may influence the results, using the pyrosequencing methylation assay in Dr. Laprise's laboratory. They found association between *ZPBP2* DNA methylation levels and asthma phenotype in five of the nine CGs (from p = 2.9E-04 to p = 7.3E-05). This associations were independent of WBC counts and higher in females in eight out of nine CGs, (p = 9.0E-04 to p = 4.4E-05) than in males three out of nine CGs (p = 6.6E-04 and p = 3.6E-04).

As in *ZPBP2*, the association between *GSDMA* promoter methylation levels and asthma phenotype was noticed in females for CG1 (hg19, chr17: 38119132). Asthmatic females had lower methylation levels in all three CGs compared with non- asthmatic (Al Tuwaijri et al., 2016).

TABLES

Table A.1 Effect of 5-aza-dC on methylation of the *ZPBP2* promoter in NuLi-1 cells (Moussette et al., 2017).

Region	*CG*	*Average methylation level in DMSO-treated cells*	*Average methylation level in 5-aza-dC treated cells*	*p-value (t-test)*	*Corresponding CG# in the SBS assay*
ZPBP2	1	9	10	0.4514	36
	2	19	25	0.1201	35
	3	11	12	0.3976	34
	4	10	8	0.1189	33
	5	8	5	**0.0343**	32
	6	36	26	**0.0035**	31
	7	10	6	**0.0197**	30
	8	11	9	**0.0237**	29
	9	11	9	0.0792	28
	10	7	5	0.0659	27
	11	11	8	0.0669	26

Data are from pyrosequencing methylation assays of three independent 5-aza-dC treatment experiments. P-values below 0.05 are shown in bold.

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Annual renewal submission

Submit date: **2016-12-02 11:56**
Project's REB approbation date: **2011-07-15**
Project number: **2012-943, 10-353, eReviews_1607**

Submitted by: **Naumova, Anna**
Nagano identifier: **10-353 GEN**
Form: **F9 - 6565**

Administration

1. **REB Decision**
   Approved

2. **Renewal Period Granted:**
   until 2017-12-06

3. **Reviewed by:**
   Terry Chow, Ph.D.
   Date processed
   2016-12-06

General information

1. **Indicate the full title of the research study**

   Genetic Characteristics of Close Relatives of Subjects with Allergic Asthma from Multiple Families from the Saguenay - Lac-St-Jean Region. Identifying the Epigenetic Signature of Asthma."

2. **If relevant, indicate the full study title in French**

3. **Indicate the name of the Principal Investigator in our institution**

   Naumova, Anna

4. **Are there local co-investigators & collaborators involved in this project?**
   No

5. **Indicate the name and the affiliation of the external collaborator(s),(if any)**

Dr. Catherine Laprise, University of Chicoutimi
Dr. Francisco Martinez, Hospital La Fe, Valencia, Spain

6. **Identify the study coordinator(s)**

Naumova, Anna

7. **Indicate each site involved in the research project**

- ☐ Other
- ☐ Montreal General Hospital
- ☐ Allan Memorial Institute
- ☐ Lachine Hospital
- ☑ Glen site
- ☐ Montreal Neurological Institute & Hospital

Glen site specifics

- ☐ Royal Victoria Hospital
- ☐ Montreal Children's Hospital
- ☐ Montreal Chest Institute
- ☐ Cedar's Cancer Center
- ☐ Center for Innovative Medicine (CIM)

Project development

1. **Study start date:**

2011-10-01

2. **Expected ending date of the study:**

- ☐ Determined date
- ☑ Undetermined date

3. **Indicate the current study status at MUHC.**

Study in progress and closed to recruitment.

4. **Add a brief statement on the study status**

Currently, we are analyzing methylation in DNA samples to determine if the sex-specific differencers in DNA methylation that we have found in the asthma-associated region are due to the sex chromosome complement or sex hormone levels.

5. **Information about the participants at this institution, since the beginning of the project**

Number of participants to be recruited according to protocol

0

6. **Since the previous REB approval (annual renewal or initial approval):**

Are there any changes to the protocol or the databank's management framework ?

No

Are there any changes to the information and consent form?

No

Are there any adverse events at this site or, for multi-center projects, an institution under the jurisdiction of our REB should be reported to the REB under section 5.2.1 of MON " SOP- REB-404001 " ?

https://muhc.ca/cae/page/standard-operating-procedures-sops

No

Has there has been any new information likely to affect the ethics of the project or influence the decision of a participant as to their participation in the project ?

No

Are there any deviations / major violations protocol (life -threatening or not meeting the inclusion / exclusion criteria)?

No

Has there has been a temporary interruption of the project?

No

Have the project results been submitted for publication, presented or published?

Yes

Please specify:

Sex- and age-dependent DNA methylation at the 17q12-q21 locus associated with childhood asthma. Naumova AK, Al Tuwaijri A, Morin A, Vaillancourt VT, Madore AM, Berlivet S, Kohan-Ghadr HR, Moussette S, Laprise C. Hum Genet. 2013 Jul;132(7):811-22. doi: 10.1007/s00439-013-1298-z. Erratum in: Hum Genet. 2013 Jul;132(7):823.
Local genotype influences DNA methylation at two asthma-associated regions, 5q31 and 17q21, in a founder effect population. Al Tuwaijri A, Gagné-Ouellet V, Madore AM, Laprise C, Naumova AK. J Med Genet. 2016 Apr;53(4):232-41.

Has the REB been notified of a conflict of interest - (apparent , potential or actual), of one or more members of the research team - that was not known when it was last approved project?

No

Do you want to bring another element to the REB's attention?

No

7. **Is there a data safety monitoring committee analyzing data on the safety and efficacy of the treatment?**

No

Signature

1. **I confirm that all information is complete & accurate.**

   First & last name of person who completed the submission

   Dr. Anna Naumova